The Journal of Laboratory and Clinical Medicine

VOL. 48 JULY, 1956 No. 1

CLINICAL AND EXPERIMENTAL

OBSERVATIONS ON A PRESUMPTIVE CASE OF PRIMARY ALDOSTERONISM

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IN 1941, Ferrebee and associates[1] reported the development of polydipsia, polyuria, hypokalemia, and intermittent paralysis in dogs receiving high doses of desoxycorticosterone. It has been known for some time that the mammalian adrenal secretes a mineralo-corticoid more potent than desoxycorticosterone. Several groups[2-6] have recently reported the demonstration and isolation of this potent mineralo-corticoid, now called aldosterone. This steroid has been reported to be one hundred twenty times as potent as desoxycorticosterone in influencing sodium and potassium metabolism.[7] Conn[8] has reported extensive observations on a case and describes the clinical features of a new disease entity which he calls primary aldosteronism. In a subsequent report[9] he relates the cure of his patient. The following is a report of another patient with the clinical characteristics of primary aldosteronism who was cured by surgical removal of an adenoma of the adrenal.

CASE REPORT

J. D., a 32-year-old Italian man, a painter by trade, was referred to the neurosurgical service of the White Memorial Hospital by Dr. Arthur Nelson on Nov. 21, 1954, because of hypertension. The patient's main complaint was excessive dryness of the throat of one year's duration. He drank two to three liters of water daily, but achieved only mo-

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Received for publication Nov. 3, 1955.

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mentary relief from his thirst. In February, 1954, he sought medical advice because of his dry mouth and throat. At that time he was found to have hypertension, for which he was given a Rauwolfia derivative and placed on a low sodium diet. This treatment failed to relieve the symptoms and hypertension. With the aid of careful questioning, the patient also recalled that during the past five months he had had a few sporadic episodes of weakness in his arms and legs which lasted for periods varying from one hour to two days. On occasion these episodes of weakness were severe enough to keep him from work. The review of systems revealed in addition only mild morning bitemporal headache for the past nine months. The patient's past history and family history were negative for known hypertension or paralysis.

On physical examination, the patient was found to be a normal-appearing white man in apparently good health. There was nothing unusual about the body build or facial characteristics. The blood pressure was 190/120 in the reclining position. The pulse rate was 70 per minute. The skin was free of acne and striae. There was slight arteriovenous nicking of the retinal vessels. The heart was normal in size with a grade 2 systolic murmur at the apical region.

There was no change in the blood pressure following intravenous injections of 5 mg. of Regitine. The blood pressure dropped from 200/120 to 120/90 one hour after 7.5 Gm. of Sodium Amytal was given intravenously. An orthocardiogram and an intravenous pyelogram were essentially normal. Table I shows the admission laboratory results.

TABLE I. INITIAL LABORATORY VALUES

BLOOD VALUES		URINARY VAUES
1. Hemoglobin	14.4 Gm.	1. Urinary protein—neg. twice
2. W.B.C.	7,100	2. pH Acid
3. N.P.N.	33-37 mg. %	3. P.S.P. 55% excreted in 2 hr.
4. Serum Na	152 meq./L.	4. 18 hr. conc. test
5. Serum K	2.1 meq./L.	Specific gravity 1.027

It was felt initially that the patient had simple essential hypertension. In reviewing the laboratory reports, we noted that the patient had a serum potassium of 2.1 meq. per liter, which was somewhat of a surprise since the patient was in such good condition, seemingly with no disease other than essential hypertension. A Smithwick-type thoracolumbar sympathectomy was performed on the left side. The left adrenal gland was examined at surgery and found to be normal.

Following surgery the patient made an uneventful recovery, and at that time repeat determinations substantiated the low serum potassium level. Further studies were instituted as a result, and these are summarized in Table II.

TABLE II. FURTHER LABORATORY TESTS

BLOOD VALUES		URINARY VALUES	
1. Serum Na	144 meq.	1. 17 ketosteroid (normal 11-27 mg./24 hr.)	17.2 mg./24 hr.
2. Serum K	1.9-2.3 meq./L.		
3. Serum Cl	97 meq./L.	2. 17 hydroxysteroid (normal 2.9-12 mg./24 hr.)	5.8 mg./24 hr.
4. CO_2 Combining power	40 meq./L.		
5. Serum Ca	9.1 mg. %	3. Uropepsin (normal 15-40 units/hour)	143 units/hour
6. Arterial pH	7.59		
7. Alveolar CO_2	47 mm. Hg	4. Potassium	61.5 meq./24 hr.
8. F.B.S.	100 mg. %	5. Sodium	122 meq./24 hr.

From these tests it became apparent that the patient had a metabolic alkalosis with hypokalemia. Since this condition seemed to involve primarily electrolyte metabolism, it was postulated that the patient had an adrenal tumor or hyperfunctioning adrenal cortex which was secreting a highly specific mineralo-corticoid. Unfortunately facilities were not available to determine the specific hormone.

In view of the known relationship between alkalosis and hypokalemia, additional studies were performed in order to determine whether or not the correction of the pH would result

in a normal serum potassium in this case (see *Discussion*). The pH of the blood was lowered to normal values by continuous intravenous infusion of 0.9 per cent ammonium chloride solution at a rate of approximately 170 ml. per hour. The serum potassium, alveolar carbon dioxide, and arterial pH were determined repeatedly until the pH reached normal limits. Fig. 1 illustrates the results of this study. It will be noted that there was no change in the serum potassium even when the pH of the blood was brought to 7.45. Within a few hours after the ammonium chloride was discontinued, the pH had returned to 7.50. For the 24 hours following this test, the blood pressure level was normal; otherwise there were no subjective or objective clinical changes. The electrocardiogram was monitored throughout the period of the test. A section of it at the beginning and at the end of the ammonium chloride administration is presented in Fig. 2.

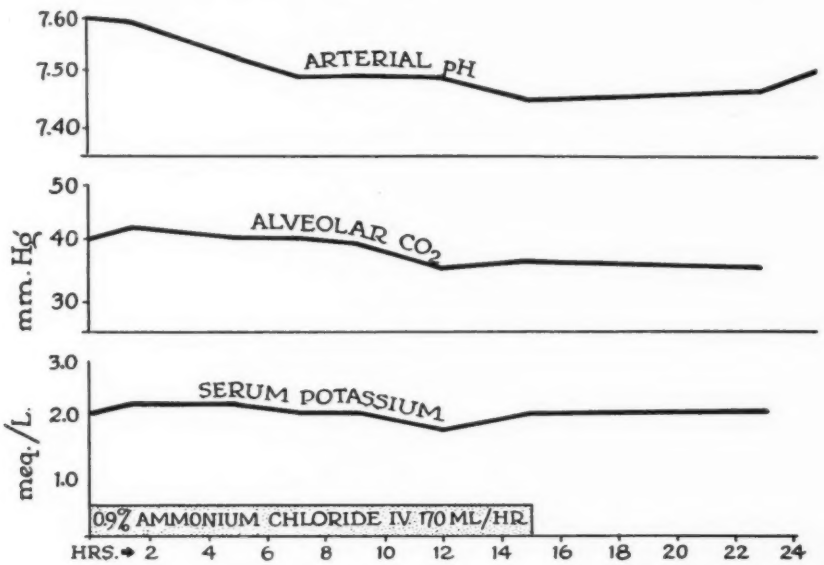

Fig. 1.—Changes in arterial pH, alveolar CO_2, and serum potassium associated with the intravenous administration of ammonium chloride (see text).

The patient was sent home on a normal diet and 4 Gm. of potassium chloride daily for eight days. The potassium chloride was then discontinued, and the patient was readmitted three days later for further study.

At the time of readmission, he still complained of dry mouth. However, he felt quite strong and had had no spells of weakness while at home. His blood pressure was 200/100 mm. Hg.

The laboratory results on readmission are presented in Table III. In addition, the alveolar CO_2 and arterial pH determined on simultaneously obtained specimens were 48 mm. Hg and 7.52, respectively. Ten milligrams of Regitine followed by 18 mg. of benzodiane had no effect on the blood pressure which remained at 185/120 mm. Hg.

TABLE III. READMISSION LABORATORY TESTS

BLOOD VALUES		URINARY VALUES	
1. Hemoglobin	12.8 Gm.	1. 17-hydroxysteroids	4.5 mg./24 hr.
2. W.B.C.	12,100	2. 17 ketogenic steroils	6.5 mg./24 hr.
3. CO_2 Combining power	40 meq./L.	(normal 9-19 mg./24 hr.)	
4. Serum sodium	146 meq./L.	3. Uropepsin	107 units/hour
5. Serum potassium	2.1 meq./L.	4. Routine analysis	
6. Serum chloride	94 meq./L.	Specific gravity 1.005	
7. Arterial pH	7.52	No albumin	

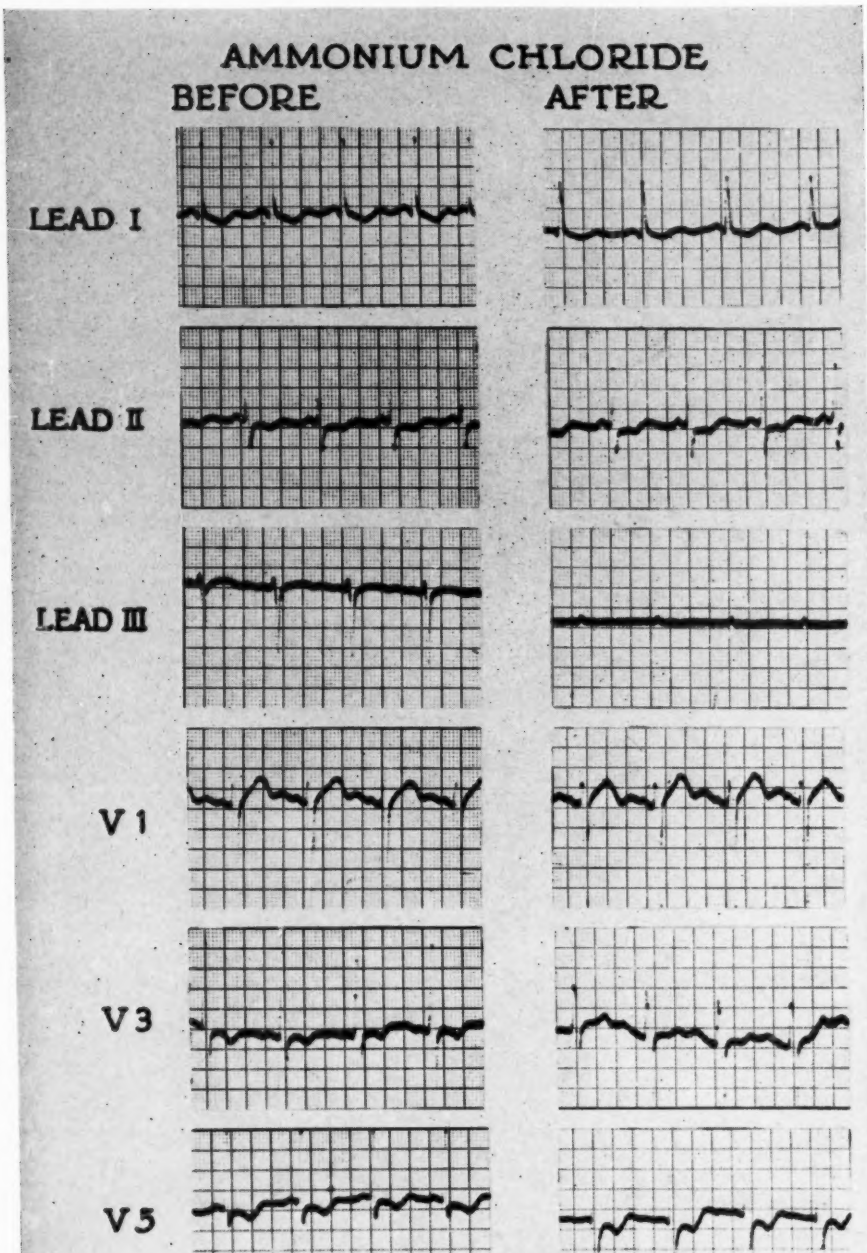

Fig. 2.—Electrocardiograms taken before and at the end of the intravenous ammonium chloride as shown in Fig. 1.

TABLE IV

TIME	24-HOUR OUTPUT 17 KETOGENIC STEROIDS	24-HOUR OUTPUT 17-HYDROXYSTEROIDS	WHITE BLOOD CELL ALKALINE PHOSPHATASE
Base line	6.5 mg./24 hr.	4.5 mg./24 hr.	46.8 (Normal 10-52)
After 25 mg. ACTH intravenously	64.4 mg./24 hr.	31.0 mg./24 hr.	86.7

The 24-hour ACTH adrenal function test, as described by Thorn and associates,[10] was done by giving the patient 25 units of ACTH intravenously over an eight-hour period. In addition to the steroid excretion rates, the white-blood-cell alkaline phosphatase level was determined by Follette.[11] Table IV gives the results of these studies. The circulating eosinophil count before ACTH was 699 per cubic millimeter, and four hours after the starting of ACTH it had dropped to 205. According to these tests the adrenal cortex seemed to be responsive.

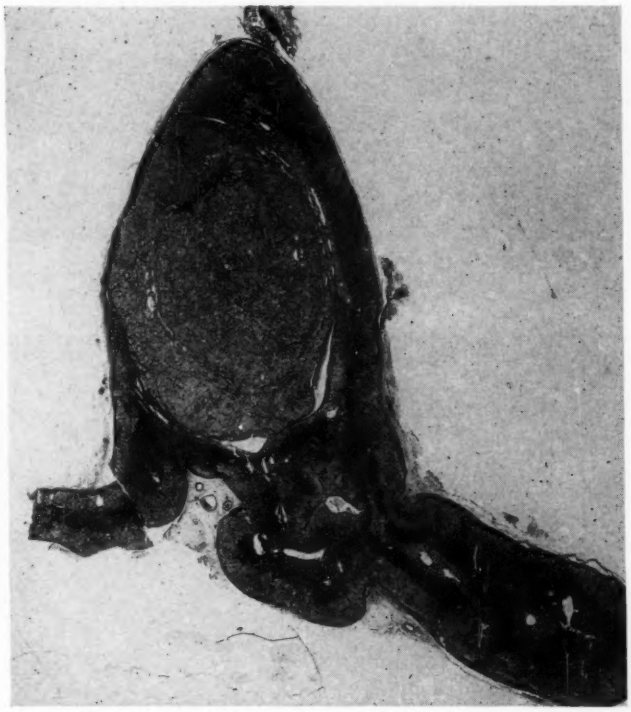

Fig. 3.—Cross section of the benign adrenal adenoma and adrenal tissue removed at surgery, demonstrating a nodule partially surrounded by adenomatous tissue with different characteristics.

The patient was prepared for surgery by giving him 25 mg. of ACTH over an eight-hour period for three days, and the right adrenal and adjacent area was explored for an adrenal cortical nodule. Since no definite nodules could be palpated or visualized, it was elected to remove approximately 75 per cent of the right adrenal gland. The right-sided sympathectomy was performed because it was not certain that removal of the functioning tissue would relieve the hypertension. The excised section of adrenal tissue, measuring 2 x 3 x 6 cm., contained a yellowish nodule of cortical tissue measuring 1 cm. in diameter (Fig. 3). On section, the specimen was found to consist of a nodule within a nodule. The cells of the centrally located nodule were arranged similar to the zona fasciculata (Fig.

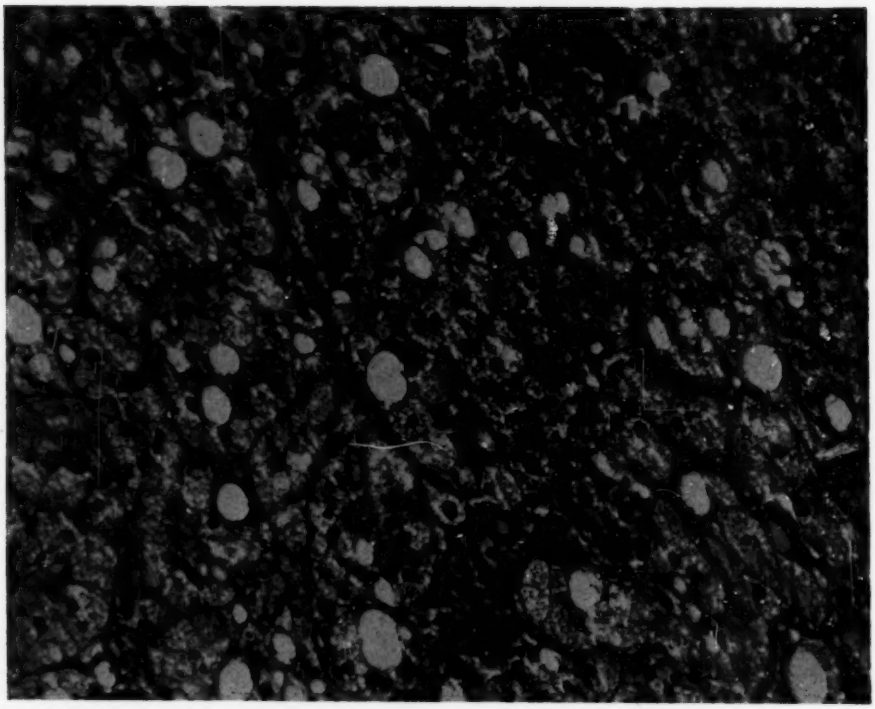

Fig. 4.—Cellular characteristics of the centrally located nodule resembling zona fasciculata (×150).

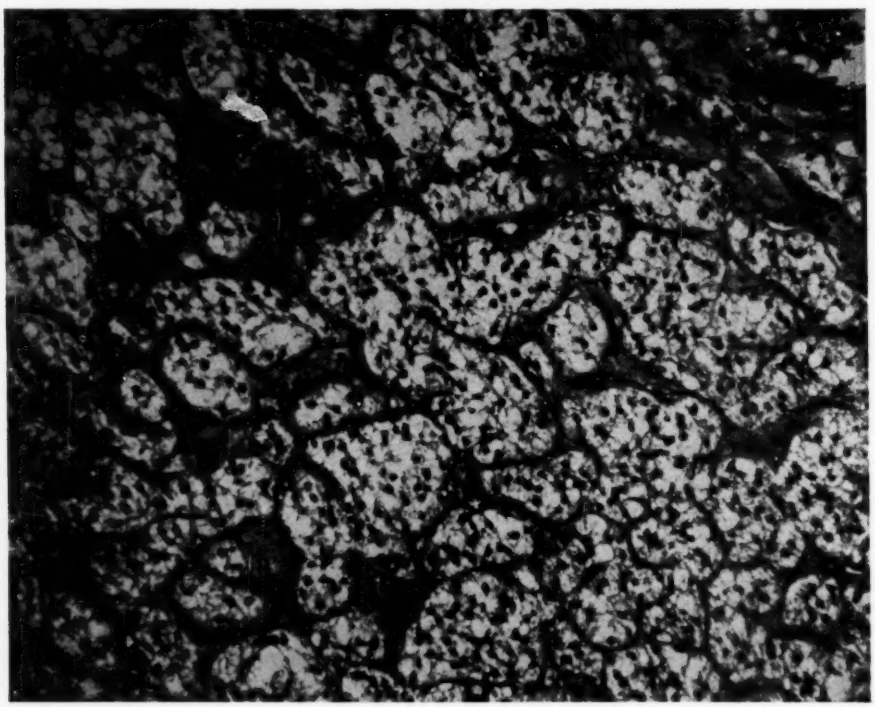

Fig. 5.—Cellular characteristics of the outer portion of the nodule resembling zona glomerulosa (×150).

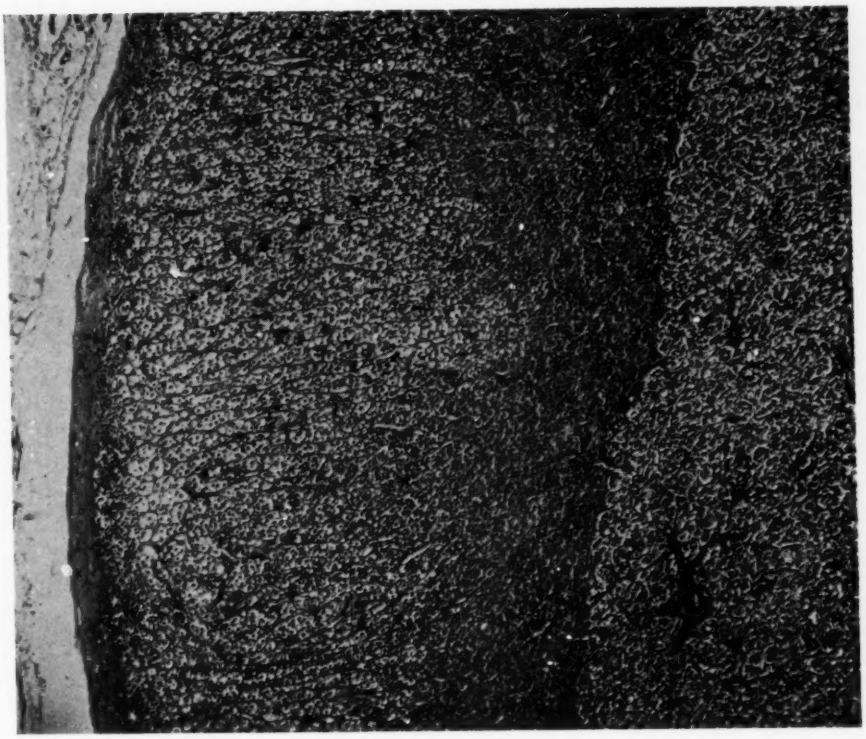

Fig. 6.—Cross section of the adrenal cortex demonstrating the three zones of normal thickness (×50).

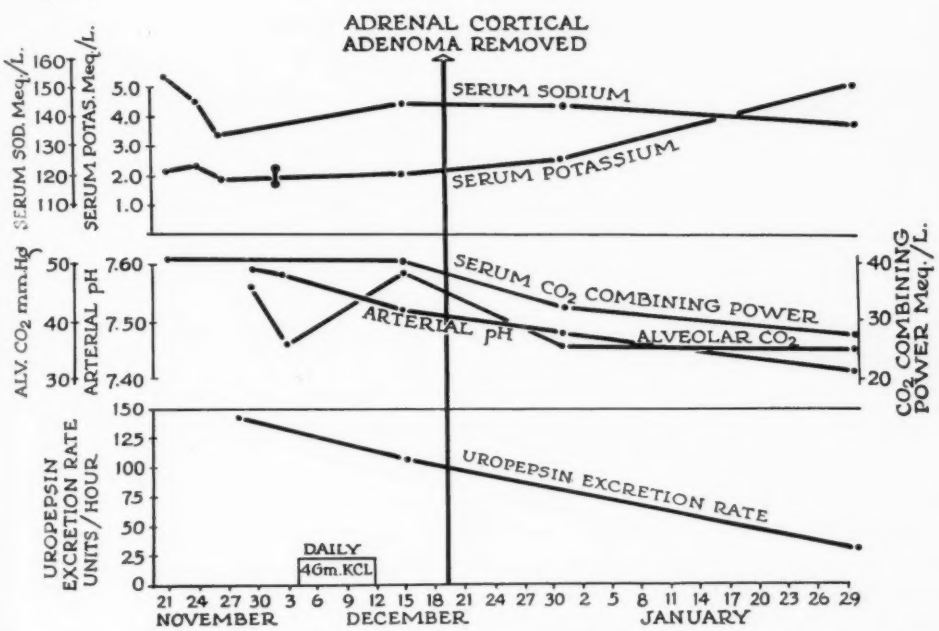

Fig. 7.—Graphic demonstration of the pertinent laboratory findings before and after removal of the adrenal cortical adenoma.

4); whereas those in the peripheral portion were arranged like the cells in the zona glomerulosa (Fig. 5). In general the cells were larger and more vacuolated than normal. No malignant cells were found.

The microscopic appearance of the adrenal gland is presented in Fig. 6. The three zones of the adrenal cortex are normal in thickness and cellular characteristics.

The first indication that the causative tissue had been removed was the statement of the patient on the second postoperative day that he no longer had a dry throat. The blood pressure has remained low since surgery with some temporary postural hypotension.

The results of the alveolar CO_2, arterial pH, serum potassium, and the CO_2 combining power determinations during the entire case study, including the follow-up studies at ten and forty days after the adenoma was removed, are presented in Fig. 7.

METHODS

The sodium and potassium concentrations were determined on a Beckman Model DU flame photometer. The alveolar CO_2 determinations were made continuously with an infrared CO_2 analyzer, as described by Collier and associates[12] while an arterial sample was obtained anaerobically. The pH was determined on this specimen immediately in a blood electrode of a Beckman Model G pH meter. The method as described by Reddy[13] was used for the determination of the 17-hydroxysteroids. The 17-ketogenic steroids were determined by the method of Norymberski.[14]

The urinary uropepsin determinations were made on timed specimens, collected under similar conditions in regard to time of day and relation to meals, and analyzed as described by West.[15] A control was used with each determination.

RESULTS

Initial Work-up.—The first studies are those of a simple essential hypertension with slight renal involvement as evidenced by the P.S.P. test, other tests of renal function being normal. The high serum sodium and low potassium suggest an adrenal cortical defect.

Further Studies.—The studies as listed in Table II confirm the hypopotassemia and demonstrate the presence of a metabolic alkalosis with high pH, alveolar CO_2, and CO_2 combining power. The glucogenic and androgenic steroids in the urine were within normal limits, but the uropepsin excretion rate was markedly elevated. The fasting blood sugar was within normal limits.

Fig. 1 demonstrates the finding that a change to normal serum pH by the administration of ammonium chloride failed to affect the serum potassium level in an acute experiment.

After two weeks on a normal diet and with an additional 28 Gm. of potassium chloride, the patient showed little change in the laboratory and clinical findings (Table III).

Fig. 7 demonstrates the extent of the hypopotassemia and metabolic alkalosis which was present before surgery and shows the follow-up studies twelve and forty-two days postoperative. These studies depict a partial return to normal by twelve days and a complete correction of the electrolyte disturbance by the last study period. The urinary uropepsin returned to normal after surgery. Subsequent determinations of uropepsin excretion have shown it to range between 32 and 40 units per hour. One year postoperatively the patient is asymp-

tomatic with a normal blood pressure. The laboratory values of the serum sodium, potassium, and CO_2 combining power have remained within normal limits.

DISCUSSION

It has been known for some time that excess desoxycorticosterone is capable of producing marked sodium retention, hypokalemia, polyuria, and intermittent paralysis in dogs.[1] Recent studies by several groups[2-6] have isolated a more potent mineralo-corticoid. This mineralo-corticoid is secreted by the adrenal cortex and does not seem to be under the control of the pituitary adrenocorticotrophic hormone.[16] It has been isolated in various conditions, particularly those with edema such as lipemic nephrosis,[17, 18] congestive heart failure,[18] toxemia of pregnancy[19, 20] hepatic cirrhosis,[20, 21] and in normal persons who are maintained on less than 11 meq. of sodium per day.[18] Luetscher[18] has pointed out that the level of the hormone output is related to the daily output of sodium rather than to the specific disease, state of hydration, or urine flow.

Recently Conn,[8, 9] has reported a new clinical syndrome in which this mineralo-corticoid, aldosterone, has been implicated. The clinical picture of his case consisted of "intermittent tetany, paresthesias, periodic severe muscular weakness and paralyses, polyuria and polydipsia, hypertension, and no edema."

The syndrome is further characterized by a severe hypokalemia, hypernatremia, alkalosis, excess amounts of sodium-retaining corticoids, and a renal tubular defect in water reabsorption. The excretion rate of the 17-ketosteroids and 17-hydroxysteroids is normal.

Except for the story of dry mouth, which was, no doubt, related to the diuresis, this case presents little in the way of significant history. There were a few episodes of weakness. Otherwise the history and findings were similar to those of the usual case of essential hypertension. The demonstration of hypokalemia, transitory hypernatremia, and metabolic alkalosis suggests a specific metabolic defect as the underlying cause for this patient's hypertension. The adrenal glands seemed to respond to pituitary stimulation normally, as evidenced by the increase in 17-hydroxysteroids and the drop in circulating eosinophils after adrenocorticotropin administration. In addition, Valentine and co-workers[11] have demonstrated that the leukocyte alkaline phosphatase level is related to pituitary-adrenal activity. The patient's initial value was normal with a normal response to ACTH. The demonstrated kidney damage was so mild that it precluded the diagnosis of a potassium-losing nephritis.

The possibility of an inadequate intake of potassium was considered and the patient was placed on a normal diet with supplementary potassium. Since too rapid repletion of chronic potassium deficiency has been reported to produce evidence of hyperkalemia,[22] the dose was maintained at 53 meq. per day for the eight-day dose period. From data reported by Schwartz and Relman,[22] the serum level of potassium should have been normal, or nearly so, after this amount was given, even though the dose was insufficient to replace the entire deficit. Conn[8] has pointed out that this syndrome resists the replacement of potassium, a fact that has been borne out in this case.

In view of the normal 17-ketosteroid, 17-ketogenic steroid, and 17-hydroxysteroid excretion rates, the elevated urinary uropepsin values take on added meaning. Uropepsin excretion rate has been found to be elevated in cases of peptic ulcer[23] and other diseases. The administration of adrenocorticotropin and cortisone has been demonstrated to produce an increased uropepsin excretion rate[24]; however, there was no evidence of an increased glucocorticoid output in this case as measured by the twenty-four-hour output of 17-hydroxy and 17-ketogenic steroids. Since the excretion rate of uropepsin in this case returned to normal after surgical removal of the adrenal cortical adenoma, this suggests that the secretion of pepsinogen by the gastric mucosa may also be under the influence of this type of mineralo-corticoid. If further studies bear this out, then uropepsin determination may become a helpful screening test along with the serum potassium, sodium, pH, and CO_2 combining power in hypertensive cases.

Moore[25] has reported the production of hypopotassemia by gastric suction until the subject is in metabolic alkalosis. The low serum potassium thus produced was then brought to normal by the administration of ammonium chloride alone. In this case, changing the pH from 7.59 to 7.45 in an acute experiment using ammonium chloride failed to influence the serum potassium level. As pointed out by Moore,[25] this may be due to the prolonged depletion of cellular stores of potassium. Another possibility is that the mineralo-corticoid had a greater influence than pH upon the potassium stores. The patient exhibited a marked resistance to correction of the metabolic alkalosis with use of ammonium chloride, as evidenced by the fact that within a few hours after discontinuation of the infusion, the pH of the blood had again become elevated.

It was unfortunate that facilities were not available for the study of the mineralo-corticoids in the urine of this patient. However, the characteristic clinical picture with the laboratory findings should leave little doubt as to the nature of the disease process. Since the left adrenal was normal on examination and only a portion of the right adrenal was removed with the adenoma, it seems reasonable to believe the adenoma was secreting the hormone. None of the three zones in the adrenal, particularly the zona fasciculata, appeared to be atrophic in this case, as was found in the case reported by Conn,[9] though the adrenocorticotropin given the three days preoperatively may have removed traces of an atrophic zone, if one existed.

In retrospect, the removal of the tumor alone would, no doubt, have cured this patient without removal of the sympathetic chain. At the time, we were unaware of a similar case in the literature having been cured by the removal of an adrenal cortical adenoma; therefore we could not be sure that the removal of the small adenoma would relieve the hypertension.

SUMMARY

A case of adrenal adenoma in a 32-year-old Italian man is presented. This patient presented himself as a case of simple essential hypertension with two unusual complaints—dry mouth and occasional episodes of muscular weakness.

The laboratory findings were characterized by severe hypokalemia and metabolic alkalosis. The 17-hydroxysteroids, 17-ketosteroid, and 17-ketogenic steroid excretion rates were within normal limits. The uropepsin rates were increased fourfold.

The symptoms, electrolyte disturbances, and increased uropepsin excretion rate were relieved by removal of a small benign adrenal cortical adenoma.

We wish to thank Mr. Stanley Weiss for the uropepsin determinations and Dr. Robert Torrey for his help on this case. We especially wish to thank Dr. Jim Follette for determining the leukocyte alkaline phosphatase and allowing us to include the results.

REFERENCES

1. Ferrebee, J. W., Parker, D., Carnes, W. H., Gerity, M. K., Atchley, D. W., and Loeb, R. F.: Certain Effects of Desoxycorticosterone, Am. J. Physiol. **135**: 230-237, 1941.
2. Simpson, S. A., Tait, J. F., Wettstein, A., Neher, R., Euw, J. Von, Schindler, O., and Reichstein, T.: Konstitution des adolesterons, des neuen mineralo-corticoids, Experentia **10**: 132-133, 1954.
3. Mattox, V. R., Mason, H. L., and Albert, A.: Isolation of a Sodium-Retaining Substance From Beef Adrenal Extract, Proc. Staff Meet., Mayo Clin. **28**: 569-576, 1953.
4. Luetscher, J. A., Jr., Neher, R., and Wettstein, A.: Isolation of a Crystalline Aldosterone From the Urine of a Nephrotic Patient, Experentia **10**: 456-458, 1954.
5. Knauff, R. E., Nielson, E. D., and Haines, W. J.: Studies on a Mineralo-Corticoid From Hog Adrenal Extract, J. Am. Chem. Soc. **75**: 4868-4869, 1953.
6. Singer, B., and Stack-Dunne, M. P.: Secretion of Aldosterone and Corticosterone by the Rat Adrenal, Nature **175**: 790, 1954.
7. Speirs, R. S., Simpson, S. A., and Tait, J. F.: Certain Biological Activities of Crystalline Electrocortin, Endocrinology **55**: 233-236, 1954.
8. Conn, J. W.: Presidential Address. Part II. Primary Aldosteronism, a New Clinical Syndrome, J. Lab. & Clin. Med. **45**: 6-17, 1955.
9. Conn, J. W.: Primary Aldosteronism, J. Lab. & Clin. Med. **45**: 661-664, 1955.
10. Thorn, G. W., Forsham, P. H., Frawley, T. F., Wilson, D. L., Renold, A. E., Fredrickson, D. S., and Jenkins, D.: Advances in the Diagnosis and Treatment of Adrenal Insufficiency, Am. J. Med. **10**: 595-611, 1951.
11. Valentine, W. N., Follette, J. H., Hardin, E. B., Beck, W. S., and Lawrence, J. S.: Studies on Leukocyte Alkaline Phosphatase Activity: Relation to Stress and Pituitary-Adrenal Activity, J. Lab. & Clin. Med. **44**: 219-228, 1954.
12. Collier, C. R., Affeldt, J. E., and Farr, A. F.: Continuous Rapid Infrared Carbon Dioxide Analysis. Fractional Sampling and Accuracy in Determining Alveolar CO_2, J. Lab. & Clin. Med. **45**: 526-539, 1955.
13. Reddy, W. J.: Modification of the Reddy-Jenkins-Thorn Method for the Estimation of 17-Hydroxycorticoids in Urine, Metabolism **3**: 489-492, 1954.
14. Norymberski, J. K., Stubles, R. D., and West, H. F.: Assessment of Adrenocortical Activity by Assay of 17-Ketogenic Steroids in Urine, Lancet **1**: 1276-1281, 1953.
15. West, P. M., Ellis, F. W., and Scott, B. L.: A Simplified Method for Determining the Excretion Rate of Uropepsin, J. Lab. & Clin. Med. **39**: 159-162, 1952.
16. Farrell, G. L., Royce, P. C., Rauschkolb, W. E., and Hirschmann, H.: Isolation and Identification of Aldosterone From Adrenal Venous Blood, Proc. Soc. Exper. Biol. & Med. **87**: 141-143, 1954.
17. Leutscher, J. A., Jr., and Johnson, B. B.: Chromatographic Separation of the Sodium-Retaining Corticoid From the Urine of Children With Nephrosis, Compared With Observations on Normal Children, J. Clin. Invest. **33**: 276-286, 1954.
18. Luetscher, J. A., Jr., and Johnson, B. B.: Observations on the Sodium-Retaining Corticoid (Aldosterone) in the Urine of Children and Adults in Relation to Sodium Balance and Edema, J. Clin. Invest. **33**: 1441-1446, 1954.
19. Gordon, E. S., Chart, J. J., Hagedorn, D., and Shipley, E. G.: Mechanism of Sodium Retention in Preeclamptic Toxemia, Obst. & Gynec. Surv. **4**: 39-50, 1954.
20. Venning, E. H., Singer, B., and Simpson, G. A.: Adrenocortical Function in Toxemia of Pregnancy, Am. J. Obst. & Gynec. **67**: 542-548, 1954.
21. Chart, J. J., and Shipley, E. S.: The Mechanism of Sodium Retention in Cirrhosis of the Liver, J. Clin. Invest. **32**: 560, 1953.

22. Schwartz, W. B., and Relman, A. S.: Metabolic and Renal Studies in Chronic Potassium Depletion Resulting From Overuse of Laxatives, J. Clin. Invest. **32**: 258-271, 1953.
23. Podore, C. J., Broh-Kahn, R. H., and Mirsky, I. A.: Uropepsin Excretion by Patients With Peptic Ulcer and Other Lesions of the Stomach, J. Clin. Invest. **27**: 834-839, 1948.
24. Spiro, H. M., Reifenstein, R. W., and Gray, S. J.: The Effect of Adrenocorticotropic Hormone Upon Uropepsin Excretion, J. LAB. & CLIN. MED. **35**: 899-910, 1950.
25. Moore, F. D.: Personal communication.

THE EFFECTS OF CORTISONE ON THE FRUCTOSE AND GLUCOSE TOLERANCE TESTS OF NORMAL MEN

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IT IS now apparent that the metabolism of fructose differs from that of glucose in several ways.[1-6] It is also well established that the administration of certain adrenal steroids may impair glucose tolerance in man.[7-11] The precise mechanism of this action is still unknown. The present study was designed primarily to determine if cortisone alters fructose tolerance as it does glucose tolerance.

METHODS

Each of ten normal men receiving a regular hospital diet providing at least 250 Gm. of carbohydrate and 3,000 calories was studied in the following manner. Control observations were made of the blood sugar levels following intravenous glucose on one occasion and intravenous fructose on another occasion. Subsequently, tolerance for each hexose was studied four hours after a single oral dose of 200 mg. of cortisone acetate. In each instance, 50 Gm. of the hexose was administered intravenously in 20 minutes as 500 ml. of a 10 per cent glucose or fructose solution. The tolerance tests were begun at 8 A.M. with the subject fasting and recumbent throughout the study. Each subject received 50 Gm. of glucose orally approximately 10 hours before each test. The two cortisone studies were performed at least 48 hours apart.†

Blood hexose was determined by the Somogyi-Nelson procedure,[13] and the method of Roe[14] was used for determining blood fructose. Urine was collected from the beginning of the infusion period to the end of the study and its sugar content determined by the semiquantitative method of Somogyi.[15]

RESULTS

1. The influence of the single oral dose of cortisone acetate upon glucose tolerance in the ten subjects is shown in Fig. 1 and Table I. The average fasting and postinfusion blood glucose levels were higher when cortisone had been given. The difference at 120, 150, and 180 minutes postinfusion were highly significant according to the "t" test using paired comparisons ($p < 0.01$). Thus the cortisone effect was most striking in its prevention of the normal hypoglycemic phase of the response to the glucose load, although the preinfusion blood sugar level was also significantly influenced by the steroid.

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Received for publication Dec. 23, 1955.

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†Eight subjects experienced epigastric discomfort during the infusion of fructose. In three of these pain occurred only with prior administration of cortisone, whereas the other five complained of some distress on both occasions when fructose was given. The etiology of this symptom has not been determined by studies designed to elucidate its mechanism.[12]

TABLE I. THE EFFECT OF A SINGLE ORAL DOSE OF CORTISONE ON THE GLUCOSE TOLERANCE TEST

SUBJECT	PRE-INFUSION		BLOOD GLUCOSE MG. PER 100 ML.											
			30 MIN.*		60 MIN.		90 MIN.		120 MIN.		150 MIN.		180 MIN.	
	A†	B‡	A	B	A	B	A	B	A	B	A	B	A	B
1	81	*89*	176	*202*	92	*112*	60	*75*	45	*71*	56	*69*	56	*85*
2	76	*73*	176	*171*	96	*96*	66	*57*	45	*41*	53	*48*	64	*59*
3	83	*98*	208	*214*	124	*132*	78	*99*	49	*83*	54	*82*	60	*83*
4	81	*94*	238	*183*	210	*134*	103	*110*	67	*104*	55	*104*	63	*103*
5	86	*90*	188	*177*	94	*97*	80	*56*	60	*59*	67	*68*	73	*75*
6	79	*85*	132	*102*	65	*78*	58	*93*	67	*99*	77	*95*	79	*97*
7	85	*93*	207	*233*	128	*161*	83	*116*	66	*92*	56	*83*	74	*86*
8	88	*94*	189	*192*	94	*113*	65	*79*	53	*81*	71	*83*	75	*88*
9	96	*159*	165	*285*	99	*240*	69	*185*	55	*154*	61	*120*	68	*88*
10	77	*100*	208	*284*	105	*218*	56	*176*	46	*147*	55	*122*	56	*103*
Mean	83.2	*97.5*	188.7	*204.3*	110.7	*138.1*	71.8	*104.6*	55.3	*93.1*	60.5	*87.4*	66.8	*86.7*
P value	<0.05		>0.1		>0.1		<0.1		<0.01		<0.01		<0.01	

*Time after completing the intravenous infusion of 500 ml. of 10 per cent glucose.
†Control.
‡After cortisone. Values in italics.

It is noted that cortisone had no significant effect on the glucose tolerance curve in Subjects 2 and 5 and resulted in markedly impaired tolerance in Subjects 9 and 10. If the remaining six subjects are considered separately, the differences remain highly significant.

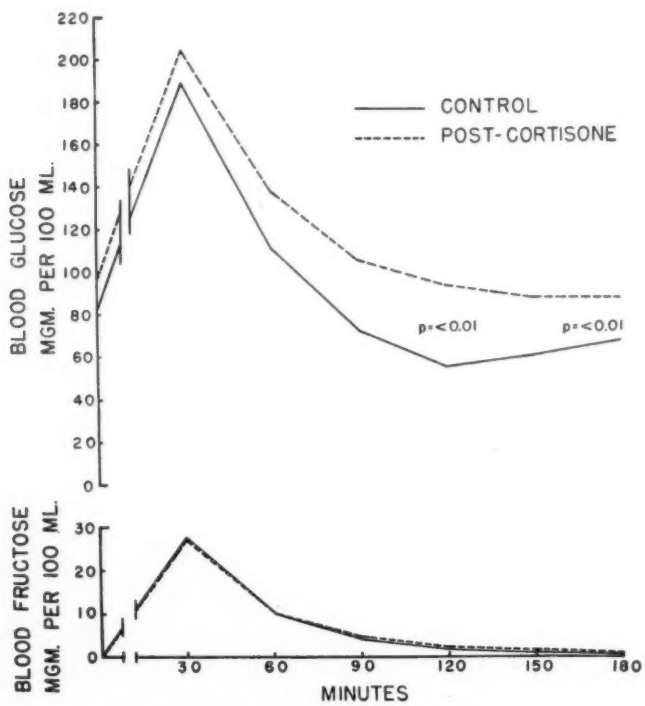

Fig. 1.—The effects of single oral dose cortisone administration on the mean glucose and fructose tolerance tests in normal men. The time in minutes after "0" refers to the time from the end of the infusion period. P values comparing the glucose curves are < 0.05 at the preinfusion time; > 0.1 at 30 and 60 minutes; < 0.1 at 90 minutes; and < 0.01 at 120, 150, and 180 minutes postinfusion.

TABLE II. THE EFFECT OF A SINGLE ORAL DOSE OF CORTISONE ON THE FRUCTOSE TOLERANCE TEST

	PRE-INFUSION		BLOOD FRUCTOSE MG. PER 100 ML.											
			30 MIN.*		60 MIN.		90 MIN.		120 MIN.		150 MIN.		180 MIN.	
SUBJECT	A†	B‡	A	B	A	B	A	B	A	B	A	B	A	B
1	0	*0*	28	*35*	14	*13*	6	*5*	3	*3*	0	*3*	0	*2*
2	0	*0*	27	*34*	8	*11*	3	*5*	2	*3*	1	*2*	1	*1*
3	0	*0*	34	*24*	13	*11*	7	*4*	2	*2*	2	*2*	1	*1*
4	0	*0*	48	*46*	18	*20*	7	*9*	3	*5*	3	*3*	2	*3*
5	0	*0*	25	*32*	10	*12*	3	*6*	2	*3*	0	*2*	1	*1*
6	0	*0*	13	*13*	4	*3*	1	*2*	1	*1*	1	*1*	1	*1*
7	0	*0*	31	*24*	11	*9*	4	*4*	1	*2*	1	*0*	1	*0*
8	0	*0*	30	*27*	11	*10*	5	*5*	2	*2*	1	*1*	1	*0*
9	0	*0*	19	*17*	5	*5*	2	*2*	0	*0*	0	*0*	0	*0*
10	0	*0*	25	*20*	8	*9*	3	*4*	2	*2*	1	*2*	1	*1*
Mean	0	*0*	28.0	*27.2*	10.2	*10.3*	4.1	*4.6*	1.8	*2.3*	1.0	*1.6*	0.9	*1.0*
P value			>0.1		>0.1		>0.1		>0.1		>0.1		>0.1	

*Time after completing the intravenous infusion of 500 ml. of 10 per cent fructose.
†Control.
‡After cortisone. Values in italics.

TABLE III. BLOOD GLUCOSE CONCENTRATION AFTER FRUCTOSE INFUSION

	PRE-INFUSION		BLOOD GLUCOSE MG. PER 100 ML.											
			30 MIN.*		60 MIN.		90 MIN.		120 MIN.		150 MIN.		180 MIN.	
SUBJECT	A†	B‡	A	B	A	B	A	B	A	B	A	B	A	B
1	83	*92*	116	*95*	87	*75*	72	*84*	64	*89*	69	*95*	95	*96*
2	75	*80*	80	*84*	67	*72*	62	*75*	68	*77*	70	*80*	69	*79*
3	88	*98*	112	*136*	82	*115*	71	*90*	76	*87*	75	*88*	72	*89*
4	78	*91*	82	*77*	73	*82*	75	*88*	80	*93*	84	*95*	83	*94*
5	80	*93*	93	*128*	69	*101*	65	*80*	68	*74*	74	*74*	83	*81*
6	72	*93*	60	*102*	75	*91*	68	*94*	78	*92*	76	*91*	83	*90*
7	88	*102*	121	*154*	99	*141*	81	*113*	77	*92*	80	*85*	93	*83*
8	89	*91*	95	*134*	72	*104*	74	*72*	81	*87*	85	*84*	85	*83*
9	107	*140*	87	*144*	66	*137*	78	*119*	76	*113*	78	*104*	79	*104*
10	78	*89*	88	*124*	76	*103*	65	*96*	62	*95*	66	*92*	67	*95*
Mean	83.8	*96.9*	93.4	*117.8*	76.6	*102.1*	71.1	*91.1*	73.0	*89.9*	75.7	*88.8*	81.0	*89.4*
P value	<0.01		<0.02		<0.01		<0.01		<0.01		<0.01		<0.01	

*Time after completing the intravenous infusion of 500 ml. of 10 per cent fructose.
†Control.
‡After cortisone. Values in italics.

TABLE IV. URINARY HEXOSE EXCRETION FOLLOWING THE INFUSION OF GLUCOSE OR FRUCTOSE*

	GLUCOSE		FRUCTOSE	
SUBJECT	CONTROL (GM.)	CORTISONE (GM.)	CONTROL (GM.)	CORTISONE (GM.)
1	4.5	5.4	3.3	4.1
2	1.5	2.5	4.2	4.6
3	4.6	5.8	4.3	5.6
4	4.0	3.0	4.0	3.5
5	3.8	4.0	4.8	4.2
6	1.2	1.4	2.8	3.2
7	1.6	2.0	2.4	3.2
8	4.6	4.4	3.2	3.9
9	4.1	10.3	3.5	3.6
10	2.4	4.5	2.2	2.1
Mean	3.2	4.3	3.4	3.8

*Excretion during 20-minute infusion period and 3 hours postinfusion period.

2. Fructose tolerance was not influenced by the administration of cortisone (Fig. 1, Table II).

3. In six subjects, the fructose infusion without cortisone resulted in a definite rise in blood glucose concentration (Table III). The group average was a rise of 10 mg. per cent without preceding cortisone and of 21 mg. per cent after cortisone (Fig. 2, Table III). The mean preinfusion blood sugar level was significantly higher after cortisone than on the control day, and a difference was sustained throughout the experiment until 180 minutes post-infusion.

4. Semiquantitative analysis of the urine sugar demonstrated that urinary excretion of hexose after glucose administration was not significantly different from that following fructose (Table IV). A marked increase in hexosuria after cortisone was observed only in Subject 9 during glucose infusion.

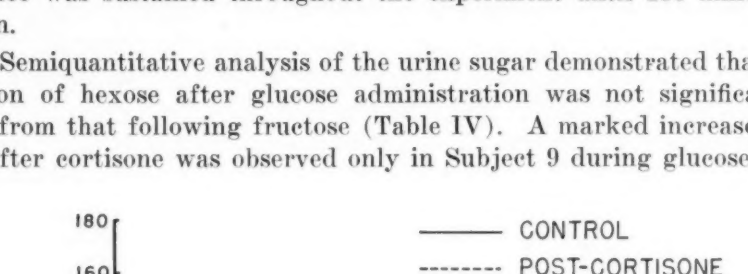

Fig. 2.—The effects of fructose infusion on the mean blood glucose concentrations in normal men with and without cortisone administration. P values comparing the glucose curves are < 0.02 at 30 minutes and < 0.01 at all other times.

DISCUSSION

The data presented agree in general with the reported "diabetogenic" action of the 11-oxysteroids in man and specifically confirm the effects of a single oral dose of cortisone upon glucose tolerance observed by Burns, Engel, and associates.[10] No such influence of the steroid upon fructose tolerance was observed even in subjects who exhibited impaired glucose tolerance after cortisone. Hein-Sekula and Siedek[16] reported an increased fructose tolerance after ACTH or cortisone. However, their experiment differs from ours since 20 Gm. of the sugar was given intravenously in 5 minutes as 50 ml. of a 40 per cent solution, and the cortisone was administered intramuscularly in suspension at the same time as the hexose was given. Since data for individual experiments are not given, statistical evaluation is not possible. Frawley,[17] who administered a fructose load during a constant infusion of hydro-

cortisone, observed greater elevation of the blood fructose than when no steroid was given. However, glucose tolerance was unaltered under the same conditions.

Several possible explanations of the dissimilar effects of cortisone on glucose and fructose tolerance warrant discussion. The differences may be considered in relation to the site of cortisone action in intermediary carbohydrate metabolism. The mechanism by which cortisone impairs glucose tolerance is not known. However, both impairment of glucose utilization and increased gluconeogenesis have been demonstrated.[18-24] Our observations permit certain inferences concerning the site of action of cortisone in the metabolic pathways of glucose utilization. It is known that the pathways for glucose and fructose in the Embden-Meyerhof scheme become identical beginning at the triose level.[6] It is suggested then that in order for cortisone to have had a selective action in the metabolism of glucose and not fructose, the hormone must act prior to the splitting of six carbon molecules into three carbon fragments in the glycolytic cycle. Another possibility is that still another pathway, such as "the hexose-monophosphate shunt" is affected by cortisone.[25, 26] However, evidence concerning hormonal control of this phosphogluconate pathway and its precise role in man is still incomplete. Another possible interpretation of these data relates to the organs affected by cortisone. If cortisone acted only upon carbohydrate utilization in the periphery, it is conceivable that it might have a selective effect on glucose disposal, since fructose appears to be metabolized largely in the liver. This explanation seems unlikely in view of studies which point to the liver as an important site of cortisone effect.[18, 23, 24]

The degree of impairment of glucose tolerance was variable, being marked in two subjects, moderate in six, and absent in two. In none was there a family history of diabetes mellitus. Prolonged observation over a period of years of individuals such as Subjects 9 and 10 may indeed support the view that they are likely to develop diabetes mellitus, but it is possible that they merely represent one part of the anticipated biologic distribution of the normal.[27]

It would appear that fructose infusion may result in a transient rise in blood glucose level and that this response is apparently magnified by cortisone. On the whole, however, the blood glucose curves on the days of the fructose infusions are very similar except that the entire curve is at a significantly higher level after cortisone administration. A hypoglycemic phase was never observed after fructose either with or without prior administration of cortisone. Thus, it appears that the hypoglycemic "dip" observed in the glucose tolerance test occurs only following a considerable elevation in the blood glucose level and is not merely the consequence of supplying exogenous carbohydrate.[28] If the hypoglycemic response is dependent upon increased endogenous insulin secretion, it may be inferred that hyperglycemia is the stimulus for this pancreatic response and that cortisone tends to interfere either with insulin production, insulin action, or both.

The literature regarding the relative degree of melituria after infusion of glucose and fructose in nondiabetic subjects is contradictory, some investigators noting less hexosuria after fructose, while Miller[2] and others[29] observe comparable quantities of urinary sugar. Our study confirms the latter observations suggesting that although the blood hexose levels are lower after fructose infusion, the lower "threshold" for fructose excretion results in the excretion of an equal amount of hexose.

The data of the present report appear to be consistent with other observations which suggest better utilization of fructose than of glucose in the postoperative period and impairment of glucose tolerance in severely ill patients in whom adrenocortical activity may be increased.[10, 29-31]

SUMMARY AND CONCLUSIONS

1. The effects of a single oral dose of 200 mg. of cortisone acetate upon intravenous fructose and glucose tolerance tests were studied in ten normal subjects.

2. Cortisone resulted in impaired glucose tolerance but did not affect fructose tolerance.

3. It is suggested that cortisone acts prior to the breakdown of six carbon molecules into three carbon fragments in the glycolytic cycle.

REFERENCES

1. Chernick, S. S., and Chaikoff, I. L.: Two Blocks in Carbohydrate Utilization in the Liver of the Diabetic Rat, J. Biol. Chem. **188:** 389, 1951.
2. Miller, M., Drucker, W. R., Owens, J. E., Craig, J. W., and Woodward, H., Jr.: Metabolism of Intravenous Fructose and Glucose in Normal and Diabetic Subjects, J. Clin. Invest. **31:** 115, 1952.
3. Smith, L. H., Jr., Ettinger, R. H., and Seligson, D.: A Comparison of the Metabolism of Fructose and Glucose in Hepatic Disease and Diabetes Mellitus, J. Clin. Invest. **32:** 273, 1953.
4. Weichselbaum, T. E., Margraf, H. W., and Elman, R.: Metabolism of Intravenously Infused Fructose in Man, Metabolism **2:** 434, 1953.
5. Renold, A. E., Hastings, A. B., and Nesbett, F. B.: Studies on Carbohydrate Metabolism in Rat Liver Slices. III. Utilization of Glucose and Fructose by Liver From Normal and Diabetic Animals, J. Biol. Chem. **209:** 687, 1954.
6. Renold, A. E., and Thorn, G. W.: Clinical Usefulness of Fructose, Am. J. Med. **19:** 163, 1955.
7. Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G.: Clinical Studies With Pituitary Adrenocorticotropin, J. Clin. Endocrinol. **8:** 15, 1948.
8. Conn, J. W., Louis, L. H., and Johnston, M. W.: Metabolism of Uric Acid, Glutathione and Nitrogen, and Excretion of "11-Oxysteroids" and 17-Ketosteroids During Induction of Diabetes in Man With Pituitary Adrenocorticotropic Hormone, J. LAB. & CLIN. MED. **34:** 255, 1949.
9. Sprague, R. G., Power, M. H., Mason, H. L., Albert, A., Mathieson, D. R., Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F.: Observations on the Physiologic Effects of Cortisone and ACTH in Man, Arch. Int. Med. **85:** 199, 1950.
10. Burns, T. W., Engel, F. L., Viau, A., Scott, J. L., Jr., Hollingsworth, D. R., and Werk, E.: Studies on the Interdependent Effects of Stress and the Adrenal Cortex on Carbohydrate Metabolism in Man, J. Clin. Invest. **32:** 781, 1953.
11. Persky, M., Linsk, J., Isaacs, M., Jenkins, J. P., Rosenbluth, M., and Kupperman, H. S.: Acute Effects of Intravenous Hydrocortisone on Glucose and Insulin Tolerances, and Levels of Serum and Urinary Inorganic Phosphorus, J. Clin. Endocrinol. **15:** 1247, 1955.
12. Saxon, L., and Papper, S.: Abdominal Pain Occurring During the Administration of Fructose Solutions. In preparation.

13a. Nelson, N.: A Photometric Adaptation of the Somogyi Method for the Determination of Glucose, J. Biol. Chem. **153:** 375, 1944.
b. Somogyi, M.: Notes on Sugar Determination, J. Biol. Chem. **195:** 19, 1952.
14. Roe, J. H., Epstein, J. H., and Goldstein, N. P.: A Photometric Method for the Determination of Inulin in Plasma and Urine, J. Biol. Chem. **178:** 839, 1949.
15. Somogyi, M.: A Rapid Method for Estimation of Urine Sugar, J. LAB. & CLIN. MED. **26:** 1220, 1941.
16. Hein-Sekula, M., und Siedek, H.: Ueber die Einwirkung von ACTH und Cortison auf den Laevuloseumsatz, Wien. klin. Wchnschr. **64:** 391, 1952.
17. Frawley, T. F.: The Role of the Adrenal Cortex in Glucose and Pyruvic Acid Metabolism in Man Including the Use of Intravenous Hydrocortisone in Acute Hypoglycemia, Ann. New York Acad. Sc. **61:** 464, 1955.
18. Long, C. N. H., Katzin, B., and Fry, E. G.: The Adrenal Cortex and Carbohydrate Metabolism, Endocrinology **26:** 309, 1940.
19. Thorn, G. W., Koepf, G. F., Lewis, R. A., and Olsen, E. F.: Carbohydrate Metabolism in Addison's Disease, J. Clin. Invest. **19:** 813, 1940.
20. Ingle, D. J.: Some Studies on the Role of the Adrenal Cortex in Organic Metabolism, Ann. New York Acad. Sc. **50:** 576, 1949.
21. Ingle, D. J., Prestrud, M. C., Nezamis, J. E., and Kuizenga, M. H.: Effect of Adrenal Cortex Extract Upon the Tolerance of the Eviscerated Rat for Intravenously Administered Glucose, Am. J. Physiol. **150:** 423, 1947.
22. Welt, I. D., and Wilhelmi, A. E.: Effect of Adrenalectomy and of Adrenocorticotrophic and Growth Hormones on the Synthesis of Fatty Acids, Yale J. Biol. & Med. **23:** 99, 1950.
23. Welt, I. D., Stetten, D., Jr., Ingle, D. J., and Morley, E. H.: Effect of Cortisone Upon Rates of Glucose Production and Oxidation in the Rat, J. Biol. Chem. **197:** 57, 1952.
24. Long, C. N. H.: The Influence of the Adrenal Cortex on Carbohydrate Metabolism, in Ciba Foundation Colloquia on Endocrinology, Vol. 6, Hormonal Factors in Carbohydrate Metabolism, Boston, 1953, Little, Brown & Co., p. 136.
25. Stadie, W. C.: The Problem of the Action of Insulin, Am. J. M. Sc. **229:** 233, 1955.
26. Stetten, D., Jr., and Topper, Y. J.: The Metabolism of Carbohydrates: A Review, Am. J. Med. **19:** 96, 1955.
27. Fajans, S. S., and Conn, J. W.: An Approach to the Prediction of Diabetes Mellitus by Modification of the Glucose Tolerance Test With Cortisone, Diabetes **3:** 296, 1954.
28. Peden, J. C., Jr., Riley, J. S., Bard, L., and Elman, R.: Blood Sugar Levels Following Intravenous Infusion of Glucose and Fructose in Adults, Metabolism **4:** 318, 1955.
29. Beal, J. M., and Smith, J. L.: The Metabolism of Fructose in Man, Surgery **36:** 243, 1954.
30. Hayes, M. A., and Brandt, R. L.: Carbohydrate Metabolism in the Immediate Post-Operative Period, Surgery **32:** 819, 1952.
31. Drucker, W. R., Miller, M., Craig, J., Jefferies, W. M., Levey, S., and Abbott, W. E.: A Comparison of the Effect of Operation on Glucose and Fructose Metabolism, Proc. Surg. Forum, Clin. Congress Am. Coll. Surgeons, Philadelphia, 1952, W. B. Saunders Co., p. 548.

ADDENDUM

Since completion of this manuscript a paper reporting failure of corticotrophin to influence fructose tolerance has appeared. (Tagnon, R. F., and Devreux, S.: Effect of Intravenous Injection of Fructose, With and Without ACTH Administration, on the Level of Blood Glucose, J. Clin. Endocrinol. & Metab. **12:** 1475, Dec. 1955.)

THE RATE OF INTRAVASCULAR EQUILIBRATION OF INTRAVENOUSLY ADMINISTERED I^{131}-LABELED ALBUMIN IN VARIOUS BODY SITES

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FOLLOWING the intravenous injection of I^{131}-labeled albumin or P^{32}- or K^{42}-labeled erythrocytes, 10 to 15 minutes are required before a relative plateau in concentration of radioactivity in arterial blood is reached.[1] However, after 1 or 2 circulations the apparent volume of distribution is already 80 per cent[2] and, at 1 to 1½ minutes, 90 per cent of the final equilibrium level.[1] This suggests that while most of the circulation is in fairly rapid equilibration, some sites require up to 10 minutes or more for complete intravascular mixing. The present studies were aimed at evaluating the rates of intravascular equilibration in different parts of the body.

METHODS

Subjects were hospitalized male patients at the Veterans Administration Hospital, Bronx, New York. Patients with evidence of cardiovascular disease were excluded from this study. Twenty-one patients received between 40 μc and 100 μc of I^{131}-labeled human serum albumin intravenously. One patient received 40 μc of K^{42}-labeled red blood cells intravenously.

In vivo assays were performed over various parts of the body with a pair of scintillation counters. The counter shields were placed in direct contact with the skin of the area of the body being assayed, the crystals being set back 5 cm. from the shield aperture. With the exception of the entire surface facing the area being studied, each crystal was completely shielded with 5 cm. of lead (Fig. 1). The counters were used interchangeably for the various studies. Each counter and scaling circuit was connected to a Streeter-Amet printing register, which recorded the cumulative counts every 30 or 60 seconds from the time of injection.

Thirty-three in vivo studies with I^{131}-labeled albumin were performed on 20 subjects. Four studies were done over calf, 2 studies over forearm, and 1 study over liver. A pair of counters was used for 5 studies over calf and forearm, 3 over head and calf, 2 over right chest and calf, 1 over liver and calf, 1 over liver and spleen, and 1 over forearm and head. A pair of counters was used for 2 studies over calf and forearm in one patient who received K^{42}-labeled red blood cells. In one patient venous blood was drawn into heparinized syringes simultaneously from an antecubital vein and a long saphenous vein at the ankle at 30- to 60-second intervals between 1½ minutes and 20 minutes following the injection of I^{131}-tagged albumin. Whole blood was counted in a well-type scintillation counter with a sensitivity of 1.0×10^6 counts per minute per microcurie I^{131}. Interval counts were corrected for background and plotted on semilogarithmic paper as per cent of the equilibrium levels against time at the midpoint of each counting interval.

From the Radioisotope Service, Veterans Administration Hospital, Bronx, N. Y.
Received for publication Dec. 10, 1955.

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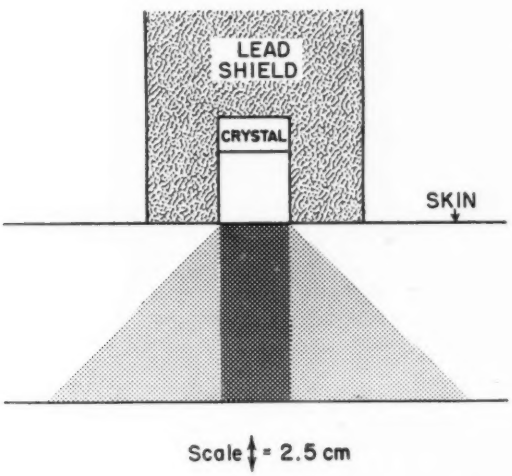

Fig. 1.—Diagram of external counting system. The inverse square law is not applicable since the crystal "sees" progressively greater areas of deeper planes of tissue. The sensitivity of the crystal for points equidistant from the crystal and containing equal concentrations of radioactivity depends upon the solid angle subtended by the crystal with each individual point.

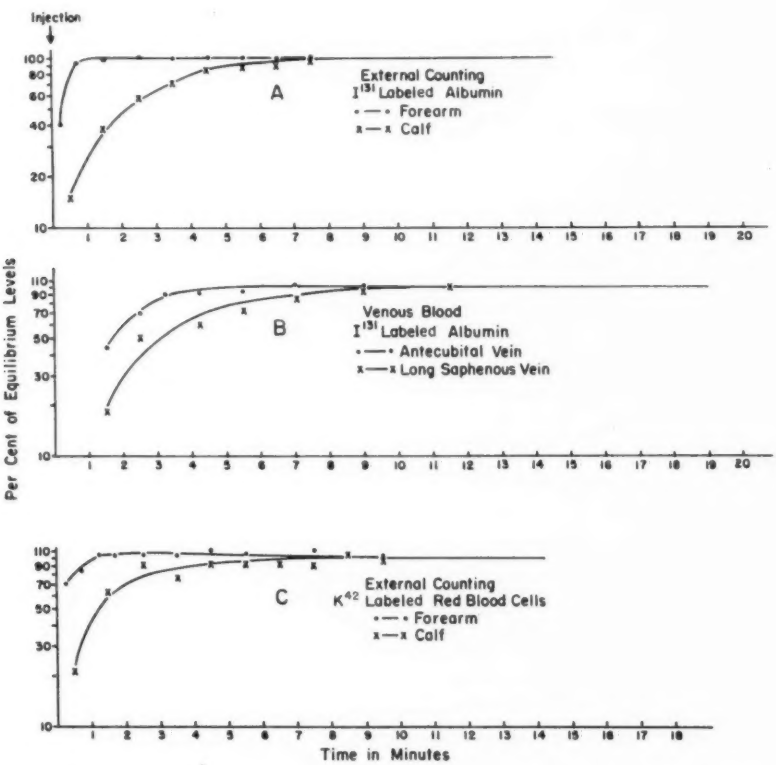

Fig. 2.—Comparison of buildup curves of radioactivity over forearm and calf using I^{131}-labeled albumin and K^{42}-labeled erythrocytes and in venous blood following the administration of I^{131}-labeled albumin.

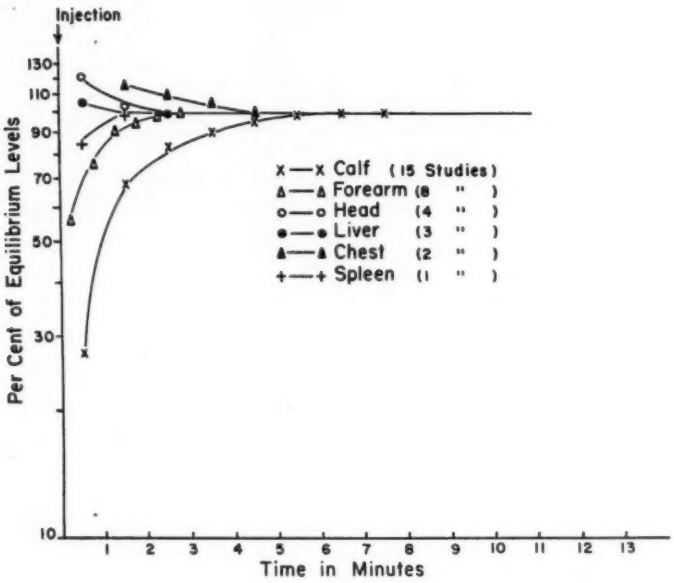

Fig. 3.—Mean buildup curves for each area studied with albumin-I^{131}. Points are expressed as per cent of equilibrium value for each region.

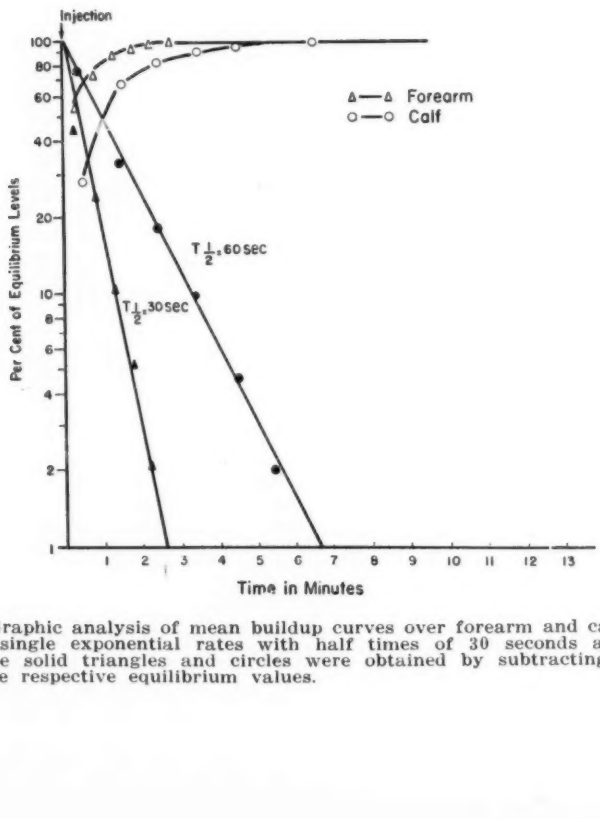

Fig. 4.—Graphic analysis of mean buildup curves over forearm and calf with albumin-I^{131} demonstrating single exponential rates with half times of 30 seconds and 60 seconds, respectively. The solid triangles and circles were obtained by subtracting the points of the curves from the respective equilibrium values.

RESULTS

Curves for equilibration in forearm and calf are presented in Fig. 2. The K^{42}-labeled red cells and I^{131}-tagged albumin curves obtained by external counting were virtually identical in general characteristics. Both revealed a considerable delay in apparent equilibration of calf blood (Fig. 2, *A* and *C*). When radioactivity in arterial blood was excluded by assaying venous blood directly, equilibration required an even longer period of time in both forearm and calf (Fig. 2, *B*). Mean buildup curves obtained by averaging points from all studies using albumin-I^{131} revealed that the concentration of radioactivity reached or exceeded equilibrium levels in 2½ minutes (range 1 to 4 minutes) and 6 minutes (range 4 to 10 minutes) in forearm and calf, respectively (Fig. 3). Radioactivity over head, right chest, and liver rose abruptly following injection and, within 30 to 90 seconds, attained values 10 to 25 per cent greater than their respective equilibrium levels before falling to a horizontal plateau (Fig. 3). In spite of the heterogeneity of processes contributing to equilibration, the approach to equilibrium in both upper and lower extremities was well described by single exponential rates with half times of 30 seconds and 60 seconds, respectively (Fig. 4).

DISCUSSION

Since there is a loss of radioactivity of only about 4 to 5 per cent from the intravascular compartment during the first hour following the intravenous injection of I^{131}-tagged albumin or K^{42}-labeled erythrocytes,[3] the buildup of radioactivity over any area reflects the rate of intravascular mixing. The rate of equilibration must depend primarily upon the total quantity of blood perfusing a region relative to its total blood content. Thus, the rapid equilibration over head and liver is consistent with the reported blood flows of 750 ml. per minute[4] and 1,500 ml. per minute,[5] respectively, perfusing organs with total weights of only 1,500 grams. On the other hand, since blood flow to resting muscle, which contains about 2 to 4 per cent of blood by weight,[6, 7] is variously estimated at between 1 and 7 ml. per 100 ml. grams per minute,[8-11] it is to be expected that equilibration in the extremities, which are largely muscular, would require several minutes. Factors responsible for differences in upper and lower extremities are not elucidated by the present studies, but it is reasonable to suggest that the importance of the hand in human function may require a greater relative blood flow through the upper extremity than through the lower extremity even at rest. The demonstration by Wisham, Yalow, and Freund[12] that the clearance of radiosodium from the biceps muscle (mean $T_{½} = 8.1$ minutes) is more rapid than from the gastrocnemius muscle (mean $T_{½} = 11.4$ minutes) is consistent with this interpretation.

Since skin has an average thickness of only 2 mm.,[13] or less than 3 per cent of the thickness of any area studied, and contains a smaller fraction of blood by weight than either muscle[6, 7] or liver,[6] its contribution to total intravascular radioactivity in the areas surveyed here is minimal when albumin-I^{131} is employed and only slightly more with K^{42}*-tagged erythrocytes. With the

*The relatively greater sensitivity for K^{42} in skin is due to β activity, which is absorbed when coming from deeper layers of tissue.

counting arrangement for the present study the decrement in radioactivity recorded from the layers of tissue at greater distances from the counter was partly compensated for since each counter "saw" progressively greater areas of the deeper planes of tissue. Since the inverse square law is not strictly applicable, skin would not be expected to account for a disproportionately greater amount of radioactivity than other tissues.

The presence of variably equilibrating pools of blood in the body requires that the more rapidly equilibrating areas attain peak values for radioactivity in excess of ultimate equilibrium levels (Figs. 2, *A* to *C*, and 3). Also, prior to complete intravascular mixing, arterial blood must contain a greater concentration of radioactivity than venous blood in slowly equilibrating areas. Thus, the increasing concentration of radioactivity in the extremities in the presence of a falling arterial concentration is explained by the fact that the arteries contain only 20 per cent of the blood in the systemic circulation while approximately 75 per cent is present in the venous system, the remainder being accounted for in capillaries.[14] The more rapid apparent rate of mixing when the external counting system is employed compared to the slower rate of equilibration observed when venous blood is assayed alone is due to the contribution of radioactivity from arterial blood.

SUMMARY AND CONCLUSIONS

1. Intravascular equilibration of I^{131}-labeled albumin and K^{42}-labeled red blood cells were studied in various parts of the body.

2. The concentration of radioactivity over head, chest, and liver attained values higher than equilibrium levels within 2 minutes and then declined to equilibrium levels. Intravascular radioactivity in forearm and calf increased gradually to reach equilibrium levels in 1 to 4 minutes and 4 to 10 minutes, respectively. These findings are consistent with the interpretation that equilibration time in various tissues is determined principally by the ratio of blood flow to blood content.

We wish to express our appreciation to Solomon A. Berson, M.D., and Rosalyn S. Yalow, Ph.D., for their helpful criticism and invaluable aid in the preparation of this manuscript. Thanks are also due Mrs. Frieda Steiner and Miss Eve Spelke, for secretarial assistance, and Mr. Paul Newman, of the Medical Illustration Department, for preparation of the figures.

REFERENCES

1. Berson, S. A., and Yalow, R. S.: The Use of K^{42} or P^{32} Labeled Erythrocytes and I^{131} Tagged Human Serum Albumin in Simultaneous Blood Volume Determinations, J. Clin. Invest. **31**: 572, 1952.
2. Bauman, A., Rothschild, M. A., Yalow, R. S., and Berson, S. A.: Transcapillary Exchange of Small Ions in the Lungs of Subjects With and Without Heart Failure. In preparation.
3. Berson, S. A.: Blood Volume in Health and Disease, Bull. New York Acad. Med. **30**: 750, 1954
4. Kety, S. S.: Circulation and Metabolism of the Human Brain in Health and Disease, Am. J. Med. **8**: 205, 1950.
5. Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J. J.: The Estimation of Hepatic Blood Flow in Man, J. Clin. Invest. **24**: 890, 1945.
6. Gibson, J. G., II, Seligman, A. M., Peacock, W. C., Aub, J. C., Fine, J., and Evans, R. D.: The Distribution of Red Cells and Plasma in Minute Vessels of the Normal Dog, Determined by Radioactive Isotopes of Iron and Iodine, J. Clin. Invest. **25**: 848, 1946.

7. Rothschild, M. A., Bauman, A., Yalow, R. S., and Berson, S. A.: Tissue Distribution of I^{131} Labeled Human Serum Albumin Following Intravenous Administration, J. Clin. Invest. **34**: 1354, 1955.
8. Kunkel, P., Stead, E. A., Jr., and Weiss, S.: Blood Flow and Vasomotor Reactions in Hand, Forearm, Foot and Calf in Response to Physical and Chemical Stimuli, J. Clin. Invest. **18**: 225, 1939.
9. Wilkins, R. W., and Eichna, L. W.: Blood Flow to the Forearm and Calf. I. Vasomotor Reaction: Role of the Sympathetic Nervous System, Bull. Johns Hopkins Hosp. **68**: 425, 1941.
10. Barcroft, H., and Edholm, O. G.: The Effect of Temperature on Blood Flow and Deep Temperature in the Human Forearm, J. Physiol. **102**: 5, 1943.
11. Andres, R., Zierler, K. L., Anderson, H. M., Sainsby, W. N., Cader, G., Ghrayyib, A. S., and Lilienthal, J. C., Jr.: Measurement of Blood Flow in the Forearm of Man: With Notes on the Theory of Indicator-dilution and on Production of Turbulence, Hemolysis, and Vasodilatation by Intravascular Injection, J. Clin. Invest. **33**: 482, 1954.
12. Wisham, L. N., Yalow, R. S., and Freund, A. J. P.: Consistency of Clearance of Radioactive Sodium From Human Muscle, Am. Heart J. **41**: 3, 1951.
13. Leider, M., and Buncke, C. M.: Physical Dimensions of the Skin, Arch. Dermat. u. Syph. **69**: 563, 1954.
14. Green, H. D.: Circulatory System: Physical Principles in Medical Physics, vol. II, Glasser, O., editor, Chicago, 1950, The Year Book Publishers, p. 228.

DIURETIC RESPONSES TO ORAL AND INTRAVENOUS WATER LOADS IN PATIENTS WITH HEPATIC CIRRHOSIS

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IMPAIRMENT of the ability to excrete an administered water load is a well-recognized abnormality in patients with hepatic cirrhosis. Although many studies of this phenomenon have been reported, data concerning the influence of the route by which water is given are meager.[1] On the basis of her own observations Ralli concluded that in patients with decompensated cirrhosis the administration of fluid, either intravenously or orally, fails to induce a normal water diuresis. Even in the "compensated" cirrhotic patient, diuresis after oral administration of water is not entirely normal.[2] Nevertheless excellent diuretic responses to intravenous infusion of glucose solutions in patients with cirrhosis are indicated by the data of others,[3-5] but comparisons with oral administration are lacking. The present communication reports paired observations in the same individual, permitting comparison of the diuretic responses to intravenous and oral water loads in a group of patients with hepatic cirrhosis.

MATERIALS AND METHODS

The subjects included 15 men in each of whom the diagnosis of Laennec's cirrhosis of the liver was made on the basis of characteristic clinical and laboratory data. In all but two, edema, ascites, or both were present. Certain pertinent findings are summarized in Table I. Four subjects free of hepatic, renal, or cardiovascular disease were similarly studied as controls.

Diuretic responses to oral and intravenous water loading on successive days were compared 18 times in the 15 patients with cirrhosis and 4 times in the control group. Eleven experiments were conducted while the patients were maintained on diets containing 10 to 35 meq. of sodium; seven while salt intake was unrestricted. The control subjects were studied after their daily sodium intake had been restricted to 10 meq. for a week. None of the subjects were allowed food or fluids after 10 P.M. on the evening prior to each procedure. The next morning, 240 ml. of water was drunk at 7 A.M. Except for three subjects who fasted throughout, a special breakfast comprising 120 ml. of orange juice, two slices of salt-free toast with jam, and 240 ml. of coffee was ingested at 7:30 A.M. Thereafter the subjects remained recumbent except for rising to void. Beginning at about 8:30 A.M. a water load of 20 ml. per kilogram was given as a 4 per cent solution of invert sugar. When ingested, the fluid was usually taken over a period of approximately 60 minutes. When infused, it was administered over a period approximately 30 minutes longer than the same subject required to ingest it. In half of the experiments, oral loading was carried out first and in the remainder intravenous loading preceded oral.*

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Received for publication Jan. 1, 1956.

*In three studies tap water was given orally and 5 per cent dextrose in water intravenously. In one case 4 per cent fructose in water was administered both intravenously and orally.

TABLE I. CLINICAL AND LABORATORY DATA IN EXPERIMENTAL AND CONTROL SUBJECTS

SUBJECT AND EXP. NO.	AGE (YR.)	WT. (KG.)	EDEMA	ASCITES	VDB (MG. %)	HGB.* (GM. %)	SERUM* TOTAL PROTEIN (GM. %)	SERUM* ALBUMIN (GM. %)	SERUM* Na (MEQ./L.)	SERUM* K (MEQ./L.)	SERUM* Cl (MEQ./L.)	SERUM* TOTAL SOLUTE (MOSM./L.)
1 (A)	61		2+	4+	3.8	12.0	5.7	2.4	136.7	4.7	104.6	
1 (B)	61	73.7	3+	4+	2.6	12.0	5.3		120.3	4.2	91.0	
2	43	56.1	1+	±	20.4	10.6	6.1	2.7	135.5	3.3	98.5	267
3 (A)	61	74.8	4+	4+	3.5	13.5	5.9		136.6	4.5	104.2	
3 (B)	62		2+	3+	2.0	12.9	6.6		142.9	4.0	105.1	
4	36	64.5	3+	4+	2.3	9.9	7.5		139.8	4.8	109.4	
5	63	88.0	3+	3+	6.2	11.8	6.3	1.8	130.5	3.8	93.1	269
6	58	46.4	0	2+	1.8	9.9	6.4		135.1	4.8	103.9	285
7	55	63.9	1+	3+	0.6	10.0	8.0	2.8	130.0	4.3	104.9	280
8	51	94.7	4+	4+	4.4	10.3	6.3		133.6	4.3	102.4	275
9 (A)	39	91.0	1+	3+	3.6	11.0	6.4	1.5	129.0	4.5	97.5	
9 (B)	39	85.0	0	0	2.8	11.7	7.2		139.4	4.6	106.0	
10	48	65.2	4+	3+	0.7	11.9	5.1		140.2	4.6	102.8	
11	36	106.4	3+	4+	1.0	14.9	6.8		137.8	4.5	100.4	279
12	33	61.0	2+	4+	2.7	10.0	6.4	1.0	133.0	3.6	94.3	
13	51	67.7	0	0	2.6	10.6	6.8		136.3	5.1	103.1	281
14	56	77.3	0	0	2.0	13.2	7.0	2.8	134.5	4.4	105.9	277
15	44	74.6	0	2+	6.3	14.2	6.7		134.2	4.0	102.9	283
16 (C)	22	82.1	0	0		14.9	7.1		134.7	4.8	100.0	281
17 (C)	27	77.7	0	0		15.8	6.8		138.3	5.0	104.1	284
18 (C)	36	83.4	0	0		14.4	6.5		139.0	4.6	98.1	287
19 (C)	22	74.2	0	0		16.0	6.3		140.0	5.2	99.9	284

*Total solute, electrolyte, total protein, and hemoglobin concentrations are initial values on day of first water load; oral route in Subjects 1(A), 2, 3(B), 4, 5, 6, 7, 13, 14, 15, 18(C), and 19(C), all others intravenous.

(A)—Initial study.

(B)—Subsequent study.

(C)—Control subject.

VDB—Serum total bilirubin.

In each study, voided urine collections were made from approximately 10 P.M. to 7:30 A.M., from 7:30 A.M. to 8:30 A.M., and thereafter when the patient felt the desire to void at intervals of 10 to 120 minutes. Blood samples, either "arterialized" venous (10 experiments) or arterial (8 experiments), were drawn just prior to water loading and about 30 minutes after the fluid had been ingested or about 10 minutes after the end of the infusion. Specimens were analyzed for sodium, potassium, chloride, and creatinine by methods previously described in publications from this laboratory.[6] Total blood hexose was determined by the Nelson photometric method using a Somogyi copper reagent[7, 8]; urine sugar was measured by the same procedure or semiquantitatively by boiling with sodium hydroxide.[9] Serum and urine osmolarities were determined cryoscopically by means of an osmometer Model B of Fiske Associates. Serum protein concentration was measured by the biuret method.[10] Change in plasma volume was calculated from hemoglobin, hematocrit, and serum total protein concentrations.[6] Free-water clearance (C_{H_2O}) was calculated as urine flow (V) minus osmolar clearance ($U_{osm.}V/P_{osm.}$).[11]

RESULTS

The experimental results in the patients with cirrhosis have been grouped as follows (Table II):

I. Severely impaired diuretic response to oral water loading; peak urine flow 3.3 ml. per minute or less (three studies in two patients).

II. Moderately or slightly impaired diuretic response to oral water loading; peak urine flow 4.9 to 9.0 ml. per minute (eight studies in seven patients).

III. Adequate diuretic response to oral water loading; peak urine flow 11.0 to 16.0 ml. per minute (seven studies in seven patients).

GROUP I.—The two patients in this category were both very ill. One had massive ascites, anasarca, and mild icterus; the other was only slightly edematous but deeply jaundiced. Each exhibited striking impairment of diuresis in response to water loading by either the oral or intravenous route. Osmolarity was not measured in the first study of Subject 1, but the urine was dilute as indicated by specific gravity determination. In the second study of this patient (two weeks before his death), there was no diuretic response whatsoever and the urine osmolarity remained well above that of plasma. This was the only instance in this series of studies in which the urine failed to become hypotonic after water loading.

Subject 2 excreted a markedly hypotonic urine after water loading by either route. The low urine flow was associated with very low rates of sodium and total solute excretion. Hence osmolar clearance was very low, as was free-water clearance. Creatinine clearances were not remarkable.

GROUP II.—Water diuresis after oral loading was moderately or slightly impaired in eight studies carried out in seven patients. The maximal rate of flow after oral hydration averaged 7.0 ml. per minute, maintained over a mean period of 57 minutes. In all but one experiment, intravenous loading was followed by a maximal diuretic rate which exceeded the response to oral hydration by 3.0 to 12.0 ml. per minute. The mean increment for the eight studies was 5.1 ml. per minute, maintained for an average of 53 minutes. In one subject the diuretic responses to oral and intravenous hydration were very similar. Urine osmolarity, measured in six of the eight studies, averaged 83 after oral and 87 mOsm. per liter after intravenous loading. The rate of sodium

excretion during peak diuresis after oral hydration averaged 64 μeq per minute for the eight experiments; after intravenous infusion the average was 164 μeq per minute. In five experiments natriuresis was greater by 53 to 363 μeq per minute after intravenous loading. Osmolar clearance was higher after intravenous loading by an average of almost 2 ml. per minute and free-water clearance by 3.3 ml. per minute. In six of the eight experiments creatinine clearance after parenteral hydration was greater by 11 to 46 ml. per minute. The mean clearances for the eight experiments were 99 ml. per minute after oral and 120 ml. per minute after intravenous loading. One study of this group is shown in detail in Fig. 1.

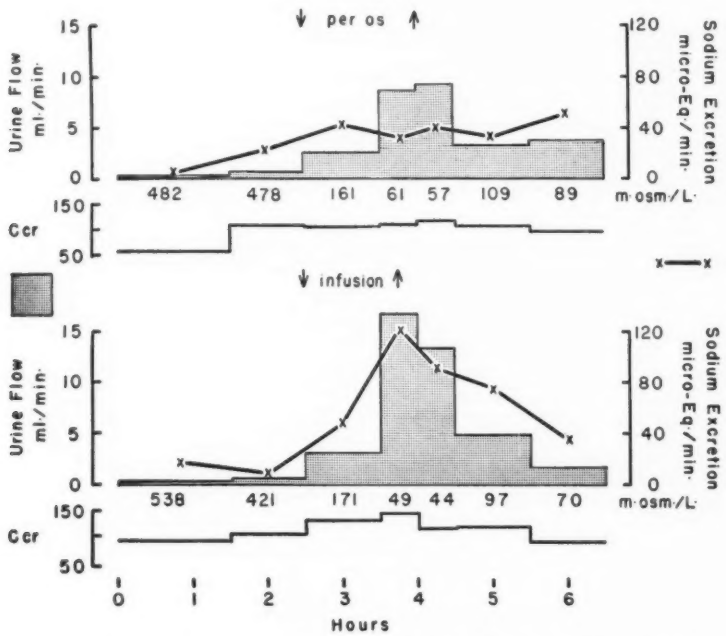

Fig. 1.—Responses to oral and intravenous water loads in Experiment 9A. Urine flow indicated by hatched blocks. The value below each block is for urine osmolarity. Rate of sodium excretion indicated by x—x. C_{cr} is endogenous creatinine clearance. This response was representative of experiments in Group II, except that parenteral hydration induced a greater reduction of urine osmolarity than did oral hydration in this experiment. In others of the group, differences in urine osmolarity after hydration by the two routes were not striking. The low initial value for C_{cr} in the oral-load study presumably reflects incomplete bladder emptying.

GROUP III.—In the seven experiments of this group maximal flow after oral loading averaged 14.0 ml. per minute. The duration of the period of peak flow averaged 57 minutes (range 46 to 65 minutes). The mean peak flow after parenteral hydration was 13.9 ml. per minute for an average duration of 51 minutes. Urine osmolarity averaged 46 and 57 mOsm. per liter after oral and intravenous loading, respectively. These mean values are well below those observed in Group II. There is some overlap but the group averages have a highly significant difference statistically ($P < 0.01$).

The paired diuretic responses differed by less than 1.5 ml. per minute in three subjects. In two subjects the peak urine flow after intravenous load-

TABLE II. MAXIMAL RENAL EXCRETORY RATES AFTER ORAL AND INTRAVENOUS LOADS

GROUP	SUBJECT AND EXP. NO.	TIME (MIN.)		FLOW (ML./MIN.)		Na (μEQ/MIN.)		K (μEQ/MIN.)		Cl (μEQ/MIN.)		TOTAL SOLUTE (μOSM/MIN.)		SOLUTE CONC. (MOSM./L.)		CREAT. CLEAR. (ML./MIN.)		C_{OSM} (ML. MIN.)		C_{H_2O} (ML./MIN.)	
		P.O.	I.V.	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.
I	1A*	60	60	3.3	3.6	1	1	19	22	9	14			(03)‡	(09)‡	142	120				
	1B	100	100	0.2	0.5	1	1	11	19	1	1	128	396	930	776	60	109	0.55§	1.59	−0.4	−1.1
	2	133	122	2.6	3.0	13	9	16	19	17	23	268	202	105	80	102	100	1.04	0.77	1.6	2.2
II	3A†	43	47	6.9	11.9	2	34	38	85	2	74			(01)‡	(01)‡	70	94				
	3B	60	60	4.9	12.0	80	443	34	63	133	443	589	1,445	120	121	61	83	2.04§	5.02	2.9	7.0
	4†	68	65	8.4	13.2	57	60	89	37	94	71			(01)‡	(01)‡	151	184				
	5	63	45	5.6	17.6	4	164	30	124	8	191	302	1,264	54	72	139	185	1.15	4.70	4.5	12.9
	6	55	54	7.0	10.9	193	285	58	74	205	293	878	1,101	112	101	101	120	3.15	4.05	3.9	6.9
	7	50	47	5.2	8.2	4	57	30	102	10	104	460	796	88	97	58	69	2.72	4.63	2.5	3.6
	8	57	43	8.8	8.2	77	98	39	44	74	109	604	698	68	85	100	106	2.25	2.60	6.5	5.6
	9A	60	60	9.0	15.1	36	107	35	38	68	120	526	706	59	47	115	130	2.03§	3.86	7.0	11.2
	Group II mean	57	53	7.0	12.1	64	164	44	71	74	176	560	1,000	83	87	99	121	2.22	4.14	4.6	7.9
III	9B	59	45	11.0	12.4	98	164	27	53	112	173	623	902	57	72	100	98	2.22	3.21	8.8	9.7
	10†	57	53	14.2	17.3	188	213	122	60	207	196					135	140				
	11	58	65	13.8	10.5	52	49	38	31	43	35	572	560	41	53	205	191	2.08	2.03	11.7	8.5
	12	65	58	11.7	7.0	52	74	40	52	4	17	395	495	34	70	116	104	1.50	1.90	10.2	5.1
	13	62	59	16.0	14.9	227	227	97	78	298	224	882	835	55	56	148	145	3.20	3.00	12.8	11.9
	14	46	30	15.2	14.3	91	100	30	37	87	98	656	671	43	47	132	127	2.42	2.46	12.8	11.8
	15	52	45	16.1	21.2	81	195	56	60	106	149	708	1,270	44	48	201	228	3.90	4.55	12.2	18.6
	Group III mean	57	51	14.0	13.9	113	146	59	53	122	127	806	789	46	57	148	148	2.55	2.36	11.3	10.9
Control	16C	45	58	15.6	14.2	19	43	29	44	21	30	578	734	37	52	139	133	2.06	2.67	13.5	11.5
	17C	61	60	11.9	13.1	13	17	103	127	31	29	621	815	52	62	164	151	2.21	2.90	9.7	10.2
	18C	63	63	11.2	12.6	21	50	63	39	25	21	666	730	59	58	134		2.36	2.57	8.8	10.0
	19C	66	58	10.6	12.1	18	23	36	38	10	9	523	576	49	48	132		1.90	2.08	8.7	10.0
	Control mean	59	60	12.4	13.0	18	36	58	62	22	22	597	714	49	55	142		2.13	2.55	10.2	10.4

*4 per cent fructose orally and intravenous.
†Tap water orally; 5 per cent glucose intravenous.
‡Specific gravity of urine—second and third digits after the decimal.
§Plasma osmolarity estimated as twice the sum serum (Na + K).

ing was greater by 3.1 and 5.1 ml. per minute, while in the remaining two patients oral hydration produced peak flows 3.3 and 4.7 ml. per minute higher.*

Except for two patients, differences in sodium excretion were small. Subject 9B excreted 66 μeq more per minute during peak flow following intravenous loading and No. 15, one of the patients with the greater diuretic response after parenteral hydration, 114 μeq more per minute. The mean sodium excretion for the whole group was 113 μeq per minute following oral hydration, 146 μeq per minute following intravenous loading. Although differences were observed in certain experiments, the average osmolar and free-water clearances after oral and intravenous loading were quite similar.

Creatinine clearances after oral and intravenous loading by the two routes were virtually the same in four of the seven experiments. In one the clearance after intravenous loading was higher by 27 ml. per minute; in two the clearances were lower by 12 and 14 ml. per minute. The latter value was obtained in the experiment complicated by the painful arterial puncture.

CONTROLS

The results in the four control studies were very similar to those in Group III patients. The very low rates of sodium excretion in the healthy subjects are consistent with the stringent restriction of dietary salt intake.

TABLE III. AVERAGE CHANGE IN BODY FLUID COMPOSITION AFTER ORAL AND INTRAVENOUS LOADING*

	Na (MEQ./L.)		K (MEQ./L.)		cl (MEQ./L.)		HEXOSE (MG. %)		TOTAL SOLUTE (MOSM./L.)		PLASMA VOLUME FROM TOT. PROTEIN (PER CENT)		PLASMA VOLUME FROM HGB. AND HCT. (PER CENT)	
	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.
Group I	−4.5	−4.5	−0.3	−0.1	−1.3	−2.1	−5	+11			+3.6	+2.7	+2.1	+3.0
Group II	−3.8	−3.5	−0.3	−0.1	−1.7	−3.1	+39	+47	−7.1	−3.3	+2.1	+2.8	+4.7	+6.5
Group III	−0.8	−3.3	−0.3	−0.4	−0.5	−1.3	−17	+13	−6.0	−4.5	+2.6	+3.8	+5.4	+4.8

*None of the changes after oral and after intravenous loading were significantly different ($P > 0.05$) as determined by the "t" test, using paired values.

CHANGES IN BODY FLUID

The data in Table III indicate that similar changes in the composition and calculated volume of the plasma were observed after water loading by either route in all three groups. The slightly greater average lowering of total solute concentration after oral loading in Groups II and III and the greater average calculated increase in plasma volume in Group II are not statistically significant. Changes in blood hexose exhibited great individual variation, related in part to difference in the initial blood glucose levels. Urinary hexose excretion was negligible in all.

DISCUSSION

The experiments described clearly demonstrate that some patients with cirrhosis attain considerably higher rates of urine flow after intravenous hy-

*A painful arterial puncture apparently inhibited the diuresis following intravenous hydration in one of the latter patients.

dration than after oral water loading. The six patients who exhibited this difference in response to oral and intravenous loading were moderately decompensated at the time of testing and exhibited some impairment of the diuretic response to oral loading. All were able to excrete moderate amounts of sodium after intravenous water loading and in only three experiments were the rates of natriuresis after oral water loading at the extremely low levels characteristic of cirrhotic patients who are forming edema and ascites maximally.[12-16] The two very ill patients in Group I had markedly impaired diuretic responses to hydration by either route and consistently manifested very low rates of sodium excretion. The seven patients who had adequate peak urine flows after both oral and parenteral loading were able to excrete considerable amounts of sodium. In three of the seven (Nos. 9B, 10, and 14), salt could be taken without restriction; in two others (Nos. 13 and 14), approximately 100 meq. of sodium per day was well tolerated, and the remaining two delivered edema during or immediately after the period of study. Rates of urine flow and free-water clearance in this group were very similar to those in the normal controls. Moreover, the rates of sodium excretion were higher in these than in the normal subjects whose sodium intakes were invariably restricted to 10 meq. daily.

Comparisons of diureses were based upon the maximal rates of urine flow during periods of approximately one hour. Differences were not ascribable to variations in the temporal course of the diuresis after oral and intravenous loading. In most instances the patients voided at intervals of 20 or 30 minutes. Diuretic responses during these shorter periods were similar to those maintained for the longer time intervals. Furthermore when the rates of urine flow were compared on the basis of even longer periods, a similar relation of the responses to oral and intravenous loading obtained. Thus in five of the seven subjects in Group II, collections were continued for 105 to 180 minutes after loading. In four of these five, the urine flow for the entire period was at least 2.0 ml. per minute higher after parenteral hydration than after water ingestion. In these four patients, the flow for the total collection period averaged 5.9 ml. per minute after oral and 8.3 ml. per minute after intravenous loading.

An apparent rise in creatinine clearance was frequently observed when diuresis took place. Interpretation is difficult since such changes are to be expected as a result of washout of urinary tract dead-space when urine flow increases.[17] Moreover the magnitude of washout artefacts is proportional to the increment and rate of change of urine flow. In only one of the seven experiments in which intravenous loading evoked a greater diuresis did C_{Cr} remain at levels during consecutive periods which were consistently higher than those observed after the oral loading (No. 4). In two others (Nos. 3A and 5), it seemed probable that augmentation in renal clearance actually occurred. In the remaining four, changes were small and seemed to be most probably related to washout. These observations suggest that the increased diuresis accomplished by intravenous loading was not necessarily related to an augmentation of filtration rate.

Urine osmolarity was generally higher in the patients with impaired diuretic responses than in the patients of Group III and the healthy controls. However only a single patient failed to excrete a hypotonic urine and that during one study when he was extremely ill.* Moreover urine concentrations well below 100 mOsm. per liter were observed despite moderate impairment of diuresis. The enhancement of diuresis after intravenous loading in Group II was associated with slight lowering of urine osmolarity in only two instances. In the others the urine was equally dilute after loading by either route or actually slightly more concentrated at the higher rate of flow. This variable relationship of concentration to flow is also indicated by the fact that both osmolar clearance and free-water clearance were higher after intravenous loading in these patients. Other observations indicate that in normal subjects under conditions of "physiologic diabetes insipidus," free-water clearance may vary directly with osmolar clearance.[19-22] It is possible therefore that in some cirrhotic patients (e.g., No. 5) impairment of water diuresis after oral loading and augmentation after intravenous loading are related almost entirely to solute excretion and not to antidiuretic hormone. The importance of antidiuretic hormone in the water retention of liver disease has been questioned by others.[5, 23]

Comparison of change in total solute with change in electrolyte excretion was possible in five experiments of the seven in which there was enhancement of diuresis after intravenous loading. The sum (Na + K + Cl) comprised 45, 65, 70, 82, and 88 per cent of the increment in total urinary solute in the five cases. Augmentation of natriuresis was very striking in several experiments. The importance of sodium excretion in normal subjects as a determinant of the magnitude of water diuresis has been previously emphasized.[24]

The data do not permit any definite conclusions concerning the nature of the stimulus for increased excretion of sodium and other solutes after intravenous loading. Since contraction of effective extracellular fluid (ECF) volume appears to be an important stimulus for sodium retention in patients with cirrhosis,[25] it is attractive to hypothesize that expansion of ECF volume provided the stimulus for salt excretion. Other studies have shown that hypotonic or isotonic expansion of ECF by parenteral infusion of sodium salts or ascitic fluid may induce natriuresis in edematous cirrhotic patients.[18] Temporary expansion of ECF volume is to be expected when isotonic or moderately hypotonic hexose solutions are given parenterally. On the other hand such expansion might well be less marked or absent when the fluid is taken orally, as suggested by Welt and Nelson.[26] In the presence of portal hypertension with delayed absorption from the gut,[27] movement of electrolytes into the salt-poor intestinal contents would result in extracellular hypotonicity without expansion of effective volume. Moreover owing to extracellular hypotonicity, water would enter cells to produce an actual fall in effective extracellular volume. A similar sequence of events has been documented when a

*Studies have been made of other patients with cirrhosis who failed to elaborate a hypotonic urine when water loaded. These patients were quite ill but in some instances were able to achieve a water diuresis after rapid expansion of circulating plasma volume and effective extracellular fluid volume by infusions of hypotonic salt solutions or ascitic fluid.[18]

hypodermoclysis of glucose solution is not absorbed promptly.[28] Furthermore, even with adequate intestinal absorption, dilution of circulating plasma proteins in portal vein blood would presumably be greater than after parenteral hydration. Lower oncotic pressure in the liver capillaries favors increased transudation and ascites formation.[29] With segregation of administered fluid in the peritoneal cavity, one would expect a sequence similar to that induced by subcutaneous or intestinal sequestration of salt-poor liquid, namely, hypotonicity, shrinkage of effective ECF volume, and cellular overhydration.[25] It seems reasonable that some such mechanisms account for the different responses to oral and intravenous loading. The failure of calculated change in plasma volume to reflect such differences is not surprising. The changes anticipated are small and the methods of measurement crude. Nevertheless Hollander and Williams,[30] who studied the immediate effects of changing the route of hydration in normal subjects undergoing water diuresis, did observe a greater change in calculated plasma volume when the intravenous route was employed.

SUMMARY

1. Diuretic responses to water loading by the oral and intravenous routes were compared in 15 patients with decompensated cirrhosis of the liver and in four subjects free of hepatic disease.

2. Two patients with marked impairment of the response to oral loading exhibited a similar response to intravenous hydration.

3. A moderately impaired response to *oral* hydration was observed in eight experiments. In seven of these, *intravenous* hydration resulted in peak flows which were higher by at least 3.0 ml. per minute.

4. In seven experiments, excellent diuresis followed oral hydration. The intravenous route did not evoke a consistently greater response in this group or in the four essentially healthy subjects.

5. In all but one experiment the urine was markedly hypotonic. Augmentation of diuresis after intravenous loading was not usually associated with further lowering of osmolarity.

6. The enhanced diuresis after intravenous loading was associated with an increase in sodium excretion, osmolar clearance, and free-water clearance.

7. The observations suggest that in cirrhotic patients moderate impairment of water diuresis after oral loading and its augmentation after intravenous loading are most clearly related to concomitant changes in excretion of sodium salts and other solutes rather than to variable inhibition of antidiuretic hormone.

The technical assistance of Miss Helen Alpert, Miss Miriam Halpin, and Miss Ellen Vaznelis is gratefully acknowledged.

Financial assistance for procurement of reprints was provided by the Gray Pharmaceutical Company, Newton, Massachusetts.

REFERENCES

1. Ralli, E. P., Leslie, S. H., Stueck, G. H., Jr., and Laken, B.: Studies of the Serum and Urine Constituents in Patients With Cirrhosis of the Liver During Water Tolerance Tests, Am. J. Med. 11: 157, 1951.

2. Papper, S., and Rosenbaum, J. D.: Abnormalities in the Excretion of Water and Sodium in "Compensated" Cirrhosis of the Liver, J. LAB. & CLIN. MED. **40**: 523, 1952.
3. White, A. G., Rubin, G., and Leiter, L.: Studies in Edema. III. The Effect of Pitressin on the Renal Excretion of Water and Electrolytes in Patients With and Without Liver Disease, J. Clin. Invest. **30**: 1287, 1951.
4. Nelson, W. P., III, and Welt, L. G.: The Effects of Pitressin on the Metabolism and Excretion of Water and Electrolytes in Normal Subjects and Patients With Cirrhosis and Ascites, J. Clin. Invest. **31**: 392, 1952.
5. Bernstein, S. H., Weston, R. E., Ross, G., Grossman, J., Hanenson, I. B., and Leiter, L.: Studies on Intravenous Water Diuresis and Nicotine and Pitressin Antidiuresis in Normal Subjects and Patients With Liver Disease, J. Clin. Invest. **32**: 422, 1953.
6. Strauss, M. B., Davis, R. K., Rosenbaum, J. D., and Rossmeisl, E. C.: "Water Diuresis" Produced During Recumbency by the Intravenous Infusion of Isotonic Saline Solution, J. Clin. Invest. **30**: 862, 1951.
7. Nelson, N.: A Photometric Adaptation of the Somogyi Method for the Determination of Glucose, J. Biol. Chem. **153**: 375, 1944.
8. Somogyi, M.: Notes on Sugar Determination, J. Biol. Chem. **195**: 19, 1952.
9. Somogyi, M.: A Rapid Method for Estimation of Urine Sugar, J. LAB. & CLIN. MED. **26**: 1220, 1941.
10. Gornall, A. G., Bardawill, C. J., and David, M. M.: Determination of Serum Proteins by Means of the Biuret Reaction, J. Biol. Chem. **177**: 751, 1949.
11. Smith, H. W.: Renal Excretion of Sodium and Water, Fed. Proc. **11**: 701, 1952.
12. Farnsworth, E. B., and Krakusin, J. S.: Electrolyte Partition in Patients With Edema of Various Origins. Qualitative and Quantitative Definition of Cations and Anions in Hepatic Cirrhosis, J. LAB. & CLIN. MED. **33**: 1545, 1948.
13. Faloon, W. W., Eckhardt, R. D., Cooper, A. M., and Davidson, C. S.: The Effect of Human Serum Albumin, Mercurial Diuretics, and a Low Sodium Diet on Sodium Excretion in Patients With Cirrhosis of the Liver, J. Clin. Invest. **28**: 595, 1949.
14. Eisenmenger, W. J., Ahrens, E. J., Jr., Blondheim, S. H., and Kunkel, H. G.: The Effect of Rigid Sodium Restriction in Patients With Cirrhosis of the Liver and Ascites, J. LAB. & CLIN. MED. **34**: 1029, 1949.
15. Eisenmenger, W. J., Blondheim, S. H., Bongiovanni, A. M., and Kunkel, H. G.: Electrolyte Studies on Patients With Cirrhosis of the Liver, J. Clin. Invest. **29**: 1491, 1950.
16. Eisenmenger, W. J.: Role of Sodium in the Formation and Control of Ascites in Patients With Cirrhosis, Ann. Int. Med. **37**: 261, 1952.
17. Smith, H. W.: The Kidney, New York, 1951, Oxford Univ. Press, p. 61.
18. Strauss, M. B., Birchard, W. H., and Saxon, L.: Tr. A. Am. Physicians **69**: 1956.
19. Welt, L. G., Young, D. T., Thorup, O. A., Jr., and Burnett, C. H.: A Study of Renal Tubular Phenomena Under the Influence of a Carbonic Anhydrase Inhibitor, J. Clin. Invest. **33**: 972, 1954.
20. Kleeman, C. R., Rubini, M. E., Lamdin, E., and Epstein, F. H.: Studies on Alcohol Diuresis. II. The Evaluation of Ethyl Alcohol as an Inhibitor of the Neurohypophysis, J. Clin. Invest. **34**: 448, 1955.
21. de Wardener, H. E., and del Greco, F.: The Influence of Solute Excretion Rate on the Production of a Hypotonic Urine in Man, Clin. Sc. **14**: 715, 1955.
22. Rosenbaum, J. D., Raisz, L. G., and Seifer, H. W.: Unpublished observations.
23. Lamdin, E., Kleeman, C. R., Rubini, M., and Epstein, F. H.: Studies on Alcohol Diuresis. III. The Response to Ethyl Alcohol in Certain Disease States Characterized by Impaired Water Tolerance, J. Clin. Invest. **35**: 1956.
24. Rosenbaum, J. D., Nelson, W. P., III, Strauss, M. B., Davis, R. K., and Rossmeisl, E. C.: Variation in the Diuretic Response to Ingested Water Related to the Renal Excretion of Solutes, J. Clin. Invest. **32**: 394, 1953.
25. Nelson, W. P., III, Rosenbaum, J. D., and Strauss, M. B.: Hyponatremia in Hepatic Cirrhosis Following Paracentesis, J. Clin. Invest. **30**: 738, 1951.
26. Welt, L. G., and Nelson, W. P., III: Excretion of Water by Normal Subjects, J. Appl. Physiol. **4**: 709, 1952.
27. McMichael, J., and Smirk, F. H.: The Effect of Experimental Portal Congestion on the Absorption and Excretion of Water, J. Path. & Bact. **37**: 81, 1933.
28. Danowski, T. S., Winkler, A. W., and Elkinton, J. R.: Biochemical and Hemodynamic Changes Following the Subcutaneous Injection of Glucose Solution, J. Clin. Invest. **26**: 887, 1947.
29. Mankin, H., and Lowell, A.: Osmotic Factors Influencing the Formation of Ascites in Patients With Cirrhosis of the Liver, J. Clin. Invest. **27**: 145, 1948.
30. Hollander, W., Jr., and Williams, T. F.: To be published.

HUMAN SERUM LIPOPROTEINS

I. Chemical Composition of Four Fractions

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THERE is relatively little published information on the chemical composition of serum lipoproteins. Neither electrophoretic nor Cohn fractionation techniques provide actual separation of the lipoproteins from other serum proteins. Chemical analyses of fractions separated by these means usually have been limited to cholesterol and lipid phosphorus determinations. Pedersen[1] was the first to take advantage of the relatively low density of lipoproteins and to float them centrifugally by increasing the density of the serum. Gofman and his co-workers[2] refined this method and defined certain classes on the basis of their flotation rates.

We have reported previously[3] a method for the determination of serum lipoproteins utilizing partial chemical analysis of several centrifugal classes. The purpose of this communication is to report more complete chemical analyses of human serum lipoprotein fractions, separated from both individual and pooled serum. A similar study in 5 men and in several animal species has been reported recently by Hillyard, Entenman, Feinberg, and Chaikoff.[4] As some of their flotation densities differed from ours, we have analyzed different fractions.

METHODS

Pools of human serum were obtained through the courtesy of the American Red Cross. All blood donor serum in excess of that required for serologic tests was pooled and delivered weekly to this laboratory. Each pool represented several hundred apparently healthy adults. The donors were not necessarily fasting but had been requested to avoid fatty food in the meal preceding bleeding. Individual serum samples were obtained from laboratory personnel either fasting or 4 hours after a fatty breakfast.

All centrifugations were carried out in the 40 rotor of the Spinco Model L ultracentrifuge at temperatures between 10 and 15° C. For the isolation of chylomicrons (Fraction 1) the serum was allowed to stand several days in the cold room. The upper chylous layer was removed and let stand several days more. The milky upper portion was then layered under an equal volume of physiologic saline (density 1.005) in several celluloid centrifuge tubes. Centrifugation was carried out as suggested by Lindgren and associates,[2] namely, for 10 minutes at 11,000 r.p.m. (9,500 × g). This resulted in an angled top layer with the consistence of butter above the now lactescent saline. The tubes were sliced through just below the butter layer in a rigid device slightly modified from that described by Randolph.[5] A needle was inserted through the hole in the cap of the tube and the saline was aspirated and discarded. The butter only was emulsified in physiologic saline and extracted as described below.

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Received for publication Dec. 16, 1955.

For the separation of lipoproteins of density less than 1.019 (Fraction 2), dry sodium chloride was added to the serum pool in appropriate amounts and the final volume adjusted with distilled water. The serum was then centrifuged for 22 hours in the 40 rotor at 40,000 r.p.m. (105,000 × g). This resulted in an opaque butter-and-creamlike layer at the top, above a wide clear zone. The tubes were sliced through the lower end of the clear zone. The supernatant material was emulsified in saline of the same density and then washed and concentrated by a second centrifugation under the same conditions. The top material was re-emulsified, dialyzed at 5° C. against physiologic saline to remove the excess salt, and extracted.

The 1.019 infranate was brought to a saline density of 1.063 by the addition of dry sodium chloride and water. Centrifugation was repeated for 22 hours at 40,000 r.p.m. This resulted in flotation of an orange-yellow layer above a clear zone. The tubes were sliced through the center of the clear zone. The supranate (Fraction 3) was washed and concentrated by a second centrifugation under the same conditions. The top material was then dialyzed and extracted.

The 1.063 infranate was then brought to a saline density of 1.21 by the addition of an appropriate amount of dry potassium bromide and the volume adjusted with water. It was centrifuged at 40,000 r.p.m. for 48 hours. This duration was found necessary to achieve complete separation of the high density lipoproteins from albumin. The tubes showed a faintly yellow upper layer above a relatively narrow clear zone. Slicing was performed as high as possible. The upper zone (Fraction 4) was concentrated for 24 hours at 40,000 r.p.m., dialyzed, and extracted.

Aliquots of the dialyzed fractions were extracted in at least 25 volumes of ethanol-acetone (1:1). A fine precipitate was achieved by forcing the material from a calibrated syringe through a 24-gauge needle into the solvent. Under these conditions, heating the mixture did not increase the yield of lipid. After standing for several hours at room temperature, the protein precipitate was packed down by centrifugation. Aliquots of the extract were analyzed for free and total cholesterol by the method of Schoenheimer and Sperry,[6] for lipid phosphorus by a modification of the method of Stewart and Hendry[7] (lipid phosphorus times 25 equals phospholipid), and for total lipid by colorimetry following dichromate oxidation.[8] As halogens react with dichromate to give a color which absorbs light at the wavelength used, a comparable aliquot of the dialysate was similarly extracted, evaporated, and treated with the dichromate reagent. An appropriate subtraction was then made. Triglyceride content was calculated by difference as described in the original paper.[8] Because this method for determining triglyceride is nonspecific and because its estimation necessitates several assumptions regarding the composition of the other lipid moieties, a more specific method was used in a few instances to confirm the presence of and to quantitate the triglyceride in Fractions 3 and 4. All the lipids except the phospholipids were extracted from the lipoprotein by a slight modification of the method of Davis.[9] They were then saponified and the glycerol was separated by partitioning between slightly acidic water and heptane. Glycerol was determined in the water phase by oxidation with sodium periodate.* The molecular ratio of formaldehyde formed to periodate reduced was 1:1, as was true for glycerol. Whereas certain other substances could yield this same ratio, the presence of glucose and galactose is excluded.

In some cases the unesterified fatty acid content of the lipoprotein fraction was determined by extracting by the method of Davis,[9] separating the fatty acids from the neutral lipids by the phase method of Borgstrom,[10] and titrating electrometrically.*

In order to determine the protein content of the lipoprotein, the precipitate formed in the extraction was washed twice with ethanol-acetone to remove lipid. In the case of Fraction 1, where relatively little protein was available, it was determined by the biuret method after dissolving in alkali. An alkali-free reagent was then added for color development. In the case of the other fractions, the precipitate was digested and the nitrogen determined by the Kjeldahl method. The factor 6.25 was used to calculate protein from nitrogen.

*We are indebted to Drs. E. Korn and R. Gordon for these determinations.

TABLE I. THE PER CENT COMPOSITION OF SERUM LIPOPROTEIN FRACTIONS

FRACTION	DENSITY	NUMBER OF POOLS	NUMBER OF INDIVIDUALS	FREE CHOLEST.	CHOLEST. ESTERS	PHOSPHOLIPID	TRIGLYCERIDE	PROTEIN	FC/TC	TC/PL	GOFMAN TERMINOLOGY
1	chylomicrons	2		3.2	6.4	7.0	81.2	2.1	0.46	1.0	
				3.0	5.7	7.1	81.4	2.8	0.47	0.90	
2	< 1.019	11		6.0 (0.4)	16.2 (1.0)	17.9 (1.1)	51.8 (2.6)	7.1 (0.8)	0.37	0.90	Sf > 10
3	1.019-1.063	16		7.5 (0.6)	39.4 (1.8)	23.1 (0.7)	9.3 (2.4)	20.7 (1.1)	0.24	1.3	Sf 0-10
			4	8.5 (0.4)	42.3 (0.8)	23.6 (0.6)	5.1 (1.2)	20.5 (0.5)	0.25	1.4	
4	1.063-1.21	11		2.0 (0.3)	17.4 (1.3)	26.1 (1.5)	8.1 (1.5)	46.4 (2.3)	0.16	0.48	High density lipoproteins
			5	2.3 (0.3)	18.5 (1.0)	26.9 (1.6)	4.6 (1.4)	47.7 (1.9)	0.17	0.50	

FC = Free cholesterol. TC = Total cholesterol. PL = Phospholipid.
The figures in parentheses represent the standard deviation from the mean.

RESULTS

Table I shows the per cent composition of the four fractions of lipoproteins. Only 2 analyses were made of Fraction 1 from pooled serum; both are given. For the other 3 fractions, mean values are given together with the standard deviation. Because of the large number of individuals in each pool the deviation is probably largely technical. As the complete removal of chylomicrons from serum was never attempted, Fraction 2 always contained some Fraction 1 material.

The most obvious difference among the fractions is the relative increase in protein content with increasing density. The most variable lipid component is the triglyceride, which varies from 81 per cent of Fraction 1 to 8 per cent of Fraction 4. The ratio of free to total cholesterol diminishes progressively with increasing density. The ratio of total cholesterol to phospholipid is greater than unity in only one fraction, Fraction 3. This does not imply that there are no other lipoprotein classes in serum with this ratio greater than one. Fraction 2 is heterogeneous, containing a wide flotation spectrum of lipoprotein classes. We have analyzed from 2 pools the lipoproteins floating between densities of 1.006 and 1.019 (Gofman's Sf 10-17), and this class also contains more cholesterol than phospholipid.

Table I also shows the composition of Fraction 3 and 4 lipoproteins of individual laboratory personnel. Although the lipid to protein ratio in these fresh serum samples is the same as in the pooled samples, there is a significant decrease in the triglyceride moiety and an increase in the cholesterol. This is true of both fractions. As most of the individuals had been fasting when the serum was drawn, it was thought that the nutritional state of the donor at the time of bleeding might affect the composition of these fractions. The sera of three individuals were therefore fractionated both in the fasting state and 4 hours after a high-fat breakfast. There were no consistent differences in the composition of either Fraction 3 or 4.

Any unesterified fatty acids that might be bound to lipoproteins, either in vivo or as an artifact, would be included in the values for triglyceride. It was thought possible that storage of the serum might have resulted in binding of fatty acids liberated by triglyceride hydrolysis. Determinations of the unesterified fatty acids in Fractions 3 and 4 from pooled serum and from fresh serum revealed no significant differences. In all cases the unesterified fatty acids accounted for less than 1 per cent of the total lipid in the molecule.

Glycerol determinations on phospholipid-free extracts of Fractions 3 and 4 from pooled and fresh serum were in good agreement with the triglyceride values as calculated, assuming that glycerol accounted for 10 per cent of the weight of the triglyceride molecule.

DISCUSSION

It must be realized that substances other than those actually determined may be present in these lipoprotein fractions. Fraction 3, for example, contains carotene. Tocopherol has been shown to accompany serum lipoproteins.[10]

Other fat-soluble vitamins may be present. Such substances would be present in negligible quantities only. Other substances, such as carbohydrates, might make up a more significant portion of the total weight. Only once was the dry weight of a lipoprotein fraction determined. The dry weight of Fraction 4 agreed very well with the total weight as determined by the chemical methods.

The composition of Fraction 1 compares favorably with the chylomicron analysis of Laurell.[12] He isolated chylomicrons by flocculation with toluidine blue from the ascitic fluid of a patient with obstruction of the thoracic duct. He found free cholesterol 1.3 per cent, cholesterol ester 6.7 per cent, phospholipid 7.3 per cent, triglyceride 84.7 per cent, and protein 1.7 per cent. The only major discrepancy is in the free to total cholesterol ratio. Gofman and associates[13] have reported, but without supporting evidence, that chylomicrons contain about 5 per cent cholesterol and that the unesterified fraction is proportionately higher than in any other lipoprotein class. They also state that chylomicrons contain about 7 per cent protein. Other attempts to characterize human chylomicrons have used methods that were either too insensitive[14] or unreliable.[15]

The denser lipoproteins have been partially characterized by several different groups. Because both electrophoretic[16, 17] and Cohn fractionation[18] techniques fail to separate the lipoproteins from other serum proteins, most of these reports concern only cholesterol/phospholipid ratios. Oncley, Gurd, and Melin,[19] who used Cohn fractionation followed by ultracentrifugation give the following composition of "beta" lipoprotein: free cholesterol 8 per cent, cholesterol esters 39 per cent, phospholipid 29 per cent, triglycerides 0 per cent, and protein 23 per cent.

The data of Hillyard and associates[4] cannot be compared directly with ours, since they fractionated between densities different from ours. Thus our Fractions 1, 2, and 3 are included in their Fraction A. Our Fraction 4 corresponds to their Fractions B and C. Their Fraction D is our 1.21 infranate.

When one adds their Fractions B and C one finds that the protein concentration varied from 53 to 60 per cent. This contrasts with our finding of 48 (± 1.9) per cent in Fraction 4 of our comparable series of 5 individuals. They also reported small amounts of cholesterol in their Fraction D, the infranate of their 1.22 density centrifugation. These findings indicate to us that their centrifugal conditions, namely 24 hours at 79,000 × g at a saline density of 1.22, were not adequate to separate these lipoproteins from albumin. We used 48 hours at 105,000 × g at a density of 1.21. Our protein content was lower and less variable, and cholesterol was not found by the Schoenheimer-Sperry method in the infranate.

Hillyard and his group encountered difficulty in extracting all the lipids in the presence of high salt concentrations. We avoided any such difficulty by dialyzing prior to extraction.

We have no explanation to offer for the higher triglyceride content of Fractions 3 and 4 in Red Cross pools as compared to fresh individual sera.

Hack[20] has recently reported that the alcohol-ether soluble phosphorus in the bottom of the centrifuge tube after prolonged centrifugation of serum is insoluble in petroleum ether and that it is probably a phosphorus-containing peptide. We have confirmed this finding in the 1.21 density infranate.[3] It thus appears that all the lipids of serum, with the exception of the unesterified fatty acids bound to albumin, float as lipoproteins at a density of 1.21.

SUMMARY

The lipoproteins of human serum have been separated into 4 fractions by the use of differential centrifugation. Each fraction has been analyzed for free and total cholesterol, lipid phosphorus, total lipid, and protein nitrogen. From these data the composition of the lipoprotein fractions has been calculated. All fractions contain all these elements but in differing proportions. Thus chylomicrons contain about 81 per cent triglyceride, but even the highest density lipoproteins may contain up to 8 per cent triglyceride. The protein content varies from 2 per cent in chylomicrons to 47 per cent in the high density lipoproteins. The ratio of free to total cholesterol is highest in chylomicrons and lowest in the high density molecules.

REFERENCES

1. Pedersen, K. O.: A Low-density Lipoprotein Appearing in Normal Human Plasma, J. Phys. and Colloid Chem. **51**: 156, 1947.
2. Lindgren, F. T., Elliott, H. A., and Gofman, J. W.: The Ultracentrifugal Characterization and Isolation of Human Blood Lipids and Lipoproteins, With Applications to the Study of Atherosclerosis, J. Phys. and Colloid Chem. **55**: 80, 1951.
3. Havel, R. J., Eder, H. A., and Bragdon, J. H.: The Distribution and Chemical Composition of Ultracentrifugally Separated Lipoproteins in Human Serum, J. Clin. Invest. **34**: 1345, 1955.
4. Hillyard, L. A., Entenman, C., Feinberg, H., and Chaikoff, I. L.: Lipide and Protein Composition of Four Fractions Accounting for Total Serum Lipoproteins, J. Biol. Chem. **214**: 79, 1955.
5. Randolph, M. L., and Ryan, M. R.: A Slicer for Sampling Liquids, Science **112**: 528, 1950.
6. Sperry, W. M., and Webb, M.: A Revision of the Schoenheimer-Sperry Method for Cholesterol Determination, J. Biol. Chem. **187**: 97, 1950.
7. Stewart, C. P., and Hendry, E. B.: The Phospholipins of Blood, Biochem. J. **29**: 1683, 1935.
8. Bragdon, J. H.: Colorimetric Determination of Blood Lipides, J. Biol. Chem. **190**: 513, 1951.
9. Davis, B. D.: The Estimation of Small Amounts of Fatty Acid in the Presence of Polyoxyethylene sorbitan Partial Fatty Acid Esters (''Tween'') and of Serum Proteins, Arch. Biochem. **15**: 351, 1947.
10. Borgstrom, B.: Investigation of Lipid Separation Methods. Separation of Cholesterol Esters, Glycerides, and Free Fatty Acids, Acta physiol. scandinav. **25**: 111, 1952.
11. Lewis, L. A., Quaife, M. L., and Page, I. H.: Lipoproteins of Serum, Carriers of Tocopherol, Am. J. Physiol. **178**: 221, 1954.
12. Laurell, C. B.: Preliminary Data on the Composition and Certain Properties of Human Chylomicrons, Scandinav. J. Clin. & Lab. Invest. **6**: 22, 1954.
13. Gofman, J. W., Lindgren, F. T., Jones, H. B., Lyon, T. P., and Strisower, B.: Lipoproteins and Atherosclerosis, J. Gerontol. **6**: 105, 1951.
14. Swank, R. L., and Wilmot, V.: Chylomicra: Their Composition and Their Fate After Intravenous Injection of Small Amounts of Heparin, Am. J. Physiol. **167**: 403, 1951.
15. Marder, L., Becker, G. H., Maizel, B., and Necheles, H.: Fat Absorption and Chylomicronemia, Gastroenterology **20**: 43, 1952.
16. Kunkel, H. G., and Slater, R. J.: Lipoprotein Patterns of Serum Obtained by Zone Electrophoresis, J. Clin. Invest. **31**: 677, 1952.

17. Nikkila, E.: Studies on the Lipid-Protein Relationships in Normal and Pathological Sera and the Effect of Heparin on Serum Lipoproteins, Scandinav. J. Clin. & Lab. Invest. **5**: Suppl. 8, 1953.
18. Russ, E. M., Eder, H. A., and Barr, D. P.: Protein-lipid Relationships in Human Plasma. I. In Normal Individuals, Am. J. Med. **11**: 468, 1951.
19. Oncley, J. L., Gurd, F. R. N., and Melin, M.: Preparation and Properties of Serum and Plasma Proteins. XXV. Composition and Properties of Human Serum Beta Lipoproteins, J. Am. Chem. Soc. **72**: 458, 1950.
20. Hack, M. H.: A Serine Phosphate in Human Serum, Fed. Proc. **14**: 222, 1955.

HUMAN SERUM LIPOPROTEINS

II. Some Effects of Their Intravenous Injection in Rats

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ONE of the earliest, if not the earliest, manifestations of atherosclerosis is the deposition of stainable lipid within the intima of the arteries or within the endocardium. Hirsch and Weinhouse[1] demonstrated that the lipid constituents of these early lesions exist in proportions comparable to those of the serum. Practically all the lipids of the serum exist as lipoprotein complexes. It therefore seemed worth-while to attempt the production of early atherosclerosis by the injection of serum lipoproteins.

Our first experiments consisted of the injection of lipoproteins from the serum of cholesterol-fed rabbits into normal rabbits. Focal microscopic deposits of sudanophilic material appeared in the aortic intima within a few hours. Using a crude fractionation technique we reported that certain lipoprotein classes appeared more pathogenic in this respect than others.[2] The rabbit, however, is not a suitable animal for this type of experiment, because spontaneous microscopic lesions occasionally occur in controls.[3] These confuse acute experiments. And, as was first shown by Bevans, Abell, and Kendall,[4] the rabbit converts intravenously administered cholesterol, even synthetic emulsions, into atherogenic lipoproteins. This makes the rabbit unsuitable for chronic experiments.

The rat was found to be a more suitable animal. Deposits of sudanophilic material have never been observed in the vessels of controls. Repeated intravenous injections of synthetic cholesterol emulsions produce no elevation in serum lipoproteins.[5] But the rat is refractory to the development of atheroma. Page and Brown,[6] who succeeded in maintaining high serum cholesterol levels by feeding both cholesterol and cholic acid to hypothyroid rats, found lipid within the aortic intima but no evidence of tissue reaction. We have confirmed this observation, but have also shown that atheroma-like lesions may appear in the endocardium. This has been demonstrated both with dietary hypercholesterolemia and following the repeated injection of lipoproteins from the serum of cholesterol-fed rabbits.[5]

The purpose of this paper is to report our observations in rats receiving intravenous injections of human serum lipoproteins. The experiments were originally designed to determine whether certain liproprotein classes might be more atherogenic than others. While this objective has not been realized, several observations have been made that are deemed worthy of reporting.

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Received for publication Dec. 16, 1955.

MATERIALS AND METHODS

Three lipoprotein fractions from pools of Red Cross serum, separated as described in the preceding paper,[7] were used. Fraction 2 contained lipoproteins with densities less than 1.019; Fraction 3 contained those with densities between 1.019 and 1.063; and Fraction 4 contained those with densities between 1.063 and 1.21. In the case of Fraction 1 (chylomicrons) quantities sufficient for injection could be obtained only from individual plasmas. Very lipemic donor plasmas were treated as described for serum pools in the preceding paper. Chemical analyses of these chylomicron preparations showed a moderate variability from those prepared from serum pools. Nothing is known of the individual donors. Some may have had extreme alimentary lipemia; others may have had idiopathic hyperlipemia.

The experimental animals were 43 male Sprague-Dawley rats weighing between 190 and 210 grams. Stock pellets and water were available to them at all times. Lipoproteins in 2 to 3 ml. of physiologic saline were injected into a tail vein. Five hours later the animals were anesthetized with intraperitoneal sodium pentobarbital and exsanguinated from the aorta. The serum was analyzed for free and total cholesterol, lipid phosphorus, and total lipid by methods described in the preceding paper. The serum lipoproteins were analyzed by one of two methods: (1) in the analytic ultracentrifuge after preliminary separation at densities of 1.063 or 1.21 (for purposes of simplicity, flotation rates have all been converted to the more familiar terminology of Gofman) or (2) by chemical analysis of four fractions separated by repeated centrifugations at increasing densities.[8]

The heart was removed, irrigated with physiologic saline, fixed in formalin, embedded in gelatin, sectioned with the freezing microtome, and stained with oil red 0 and hematoxylin. In representative animals receiving Fractions 2, 3, and 4, lung, spleen, liver, and kidney also were examined.

Three control rats each received 3 ml. of dialyzed 1.21 density infranate. They thus received a relatively large dose of human serum proteins free of lipoproteins.

The instability of isolated serum lipoproteins is well known. Ray, Davisson, and Crespi[9] have recently shown that the degradative process is oxidative in nature and is catalyzed by copper. Attempts were made to keep the time from lipoprotein separation to injection at a minimum. Dialysis usually took place the day of injection but occasionally during the preceding night.

RESULTS

Fig. 1 shows the serum total lipid levels 5 hours following lipoprotein injection plotted against the injected dose of total lipid. In the case of rats receiving Fraction 1, the serum levels were rather independent of the dose. In rats receiving Fractions 2, 3, and 4, the serum total lipid was proportional to the quantity of lipid injected. At any given dose, however, this value varied considerably among rats receiving different fractions. It can be seen, for example, that at a dosage level of 500 mg. of total lipid per kilogram of body weight the sera of rats receiving chylomicrons contain a normal amount of lipid; the sera of rats receiving Fraction 2 show lipid levels 3 times normal; those of rats receiving Fraction 3 have 5 times their normal lipid content; and the sera of rats receiving Fraction 4 contain even more.

The picture is complicated by the appearance in the recipient's serum of lipoprotein classes other than those injected. Fig. 2 shows a schematic drawing of analytic ultracentrifuge patterns of the 4 fractions and of the serum of rats receiving these same fractions. Each pattern is taken from a representative analysis. The patterns are only semiquantitative. Table I shows similar results determined by the more quantitative method of fractional centrifugation.

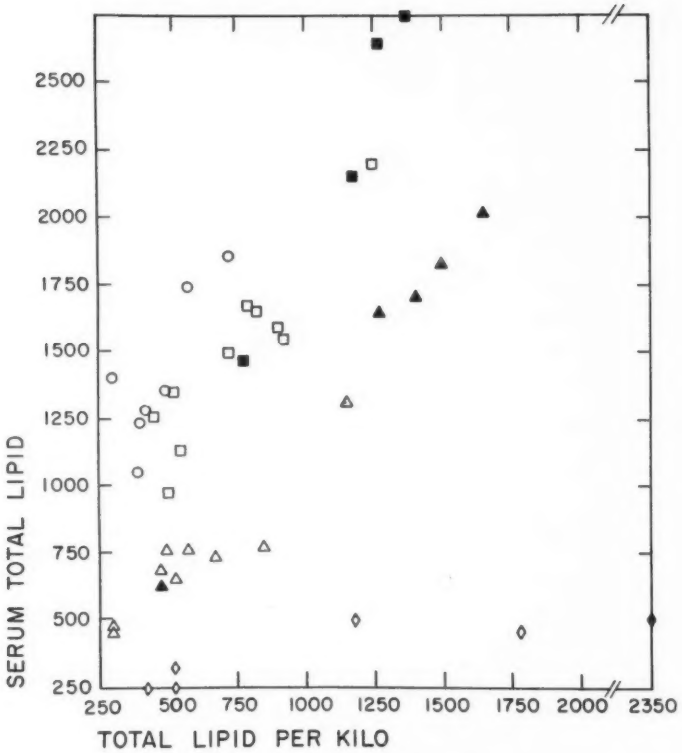

Fig. 1.—Relation of serum total lipid levels, in milligrams per 100 ml., in individual rats plotted against the total lipid injected per kilogram of body weight. ◇'s represent rats receiving Fraction 1; △'s represent rats receiving Fraction 2; □'s represent rats receiving Fraction 3; and O's represent rats receiving Fraction 4. The solid symbols indicate animals which showed focal deposits of lipid within the endocardium.

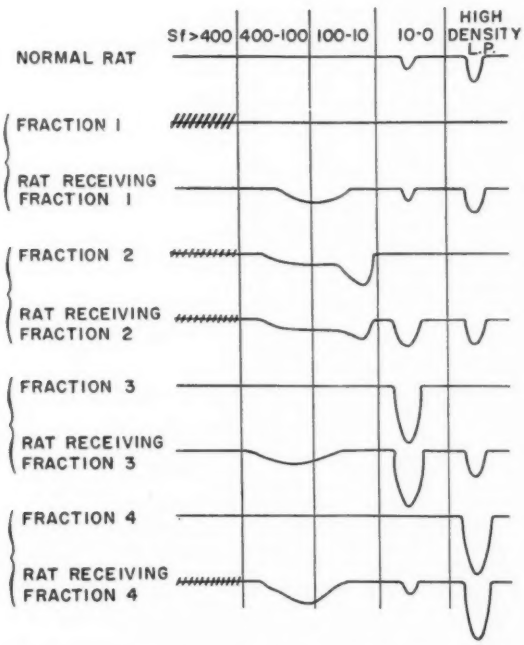

Fig. 2.—Schematic ultracentrifuge patterns of individual fractions and of the rat's serum 5 hours after intravenous injection. These rats received relatively large but not comparable doses. The crosshatching represents light scattered by the higher Sf classes. Depression below the base line represents, in a semiquantitative way, the amounts of lower Sf classes.

TABLE I. COMPOSITION OF FRACTIONS OF RAT SERUM FIVE HOURS AFTER INTRAVENOUS INJECTION OF COMPARABLE HUMAN FRACTIONS

	MG. IN FRACTION PER 100 ML. OF SERUM													
	FRACTION 2			FRACTION 3			FRACTION 4			1.21 INFRANATE			% RECOVERY	
	TC	PL	PR	TC	PL	PR	TC	PL	PR	TC	PL		TC	PL
Normal rat pool (nonfasting)	5	15	7	8	12	11	30	40	76	0	44		92	100
Rats receiving Fraction 2														
580 mg. TL/K	49	77	25	43	47	23	35	81	85	0	35		89	97
98 mg. TC/K														
1,160 mg. TL/K	206	193	76	55	54	33	28	63	89	0	31		104	94
197 mg. TC/K														
Rats receiving Fraction 3														
780 mg. TL/K	28	69	35	451	345	267	15	41	82	0	46		100	103
304 mg. TC/K														
817 mg. TL/K	41	74		423	319		15	36		0	45		99	98
318 mg. TC/K														
Rats receiving Fraction 4														
310 mg. TL/K	50	118	42	20	40	28	120	274	370	0	36		97	98
68 mg. TC/K														
733 mg. TL/K	65	130	50	19	36	32	241	515	715	0	46		95	98
161 mg. TC/K														

TC = Total cholesterol.
PL = Phospholipid.
PR = Protein.
TL/K = Total lipid per kilogram of body weight.

It shows the total cholesterol, phospholipid, and protein content of the several lipoprotein fractions of the recipient's serum. The serum of no rat receiving Fraction 1 was so analyzed.

Rats receiving Fraction 1 (chylomicrons) even in the largest doses, showed clear serum 5 hours later. With the larger doses significant amounts of lipoproteins with Sf values between 30 and 300 appeared. There was no significant increase in the Sf 0 to 10 class or in the high density lipoproteins.

Rats receiving Fraction 2 in doses over 500 mg. total lipid per kilogram showed some residual lactescence of the serum. Both analytic and fractional ultracentrifugation revealed large amounts of liproproteins of densities comparable to those injected. In addition there was a significant increase in Fraction 3. Fraction 4 showed an increase in phospholipid content.

Rats receiving Fraction 3, even in the highest doses, showed no increased lactescence of the serum 5 hours later. Both centrifugal methods of analysis showed some increase in Sf 40 to 400 (Fraction 2) lipoproteins. Large amounts of lipoproteins of the same densities as those injected were present. Fraction 4 showed a decrease in cholesterol content.

In rats that received the higher doses of Fraction 4, the serum became definitely lactescent, indicating the presence of very low-density lipoproteins. The analytic ultracentrifuge showed light scattering during acceleration and a large peak between Sf 50 and 300. Fractional ultracentrifugation showed almost a tenfold increase in Fraction 2 lipoproteins over the normal level. There was also a two- to threefold increase in Fraction 3 lipoproteins and a large amount of material of the same density as that injected. One rat received Fraction 4 lipoproteins separated from a large pool of rat serum. The dose was 255 mg. total lipid per kilogram. The disappearance rate was the same as occurred with human Fraction 4. The recipient's serum showed a sixfold increase in Fraction 3 lipoproteins but no increase in Fraction 2.

Table I shows that in no instance was there a significant change in the alcohol-acetone soluble phosphorus in the 1.21 infranate. Table I also shows that the recovery of cholesterol and phospholipid from the several fractions closely approximated that found in the whole serum.

None of the 3 control rats which received the dialyzed 1.21 infranate showed any elevation in serum lipid levels.

In some of the experimental animals histologic examination of the heart revealed on the mitral or aortic cusps small scattered foci in which endothelial and connective tissue cells contained fine granules of sudanophilic material. In some animals the medium-sized coronary arteries showed similar sudanophilic granules within the wall. Here a more diffuse distribution suggested an extracellular location. There was no evidence of any cellular reaction. Examination under polarized light showed no doubly refractile crystals. The animals which exhibited these lesions are represented by solid symbols in Fig. 1. The animal receiving the highest dose of Fraction 1 showed lesions. Several animals receiving large doses of Fractions 2 and 3 showed lesions, whereas no animal receiving Fraction 4 did.

Sections of liver, spleen, kidney, and lungs from animals receiving Fractions 2, 3, and 4 showed no abnormalities other than increased amounts of sudanophilic material in the liver cells of animals receiving Fraction 2.

DISCUSSION

Our observations are subject to the criticism that the injected lipoproteins came from a different species. The injections never evoked any signs of respiratory or circulatory distress. Control rats, which received far greater quantities of foreign protein in the form of 1.21 infranate, had normal serum lipid levels 5 hours later. We believe therefore that the changes observed in the recipients' lipoprotein patterns are specific effects of the injected lipoprotein fractions and that these changes may have some significance in lipoprotein metabolism. The one rat which received rat serum lipoproteins, in the form of Fraction 4, resembled those receiving the comparable human fraction in showing an increase in Fraction 3 but differed from them in showing no increase in Fraction 2. This rat received, however, the smallest dose on a total lipid per kilogram basis.

The data presented in Fig. 1 show that Fraction 1, composed of chylomicrons, is removed very rapidly from the blood stream. The rate of disappearance of the other fractions may be estimated from the data in Table I. A comparison between the total cholesterol injected and the total cholesterol in the same fraction less the normal level of that fraction reveals that Fraction 2 disappears more rapidly than Fraction 3 or 4, which appear to have rather similar clearance rates. Pierce[10] separated certain lipoprotein classes from the serum of cholesterol-fed and alloxanized rabbits and injected them into control rabbits. He then followed in the analytic ultracentrifuge the lipoprotein pattern of the recipient's serum at different time intervals. He found that the lower the density of a class, the more rapidly it disappeared.

The conversion of low-density lipoproteins to those of higher density has been reported to occur in vivo following fatty meals[11] and following heparin injection.[12] Pierce[10] also found this conversion of low density classes to higher, with the product always present in smaller amounts.

It is noteworthy that the phospholipid content of Fraction 4 increased following the injection of Fraction 2. This is the same phenomenon that we reported as occurring following the intravenous injection of heparin in humans with alimentary lipemia.[13] We have no explanation either for this shift or for the decrease in the cholesterol content of Fraction 4 following the injection of Fraction 3.

The appearance of low-density lipoproteins following the injection of those of higher density was unexpected. It was most noticeable following the injection of Fraction 4. The increase in lipoproteins of Fraction 2 could not have been the result of technical errors under these conditions. There may have been a conversion of Fraction 4 lipoproteins to those of Fraction 2. Alternatively, the large quantity of high density lipoproteins injected may have in some way blocked the normal mechanisms that remove low-density lipoproteins, which are presumably involved in fat transport, from the blood. If the latter is the

correct interpretation, the situation would be analogous to the accumulation of low-density lipoproteins in the serum of fasting rats injected with antiheparin agents.[14]

Pierce claimed that the injection of Sf 5 to 15, comparable to our Fraction 3, was not followed by the appearance of higher Sf classes, except after repeated bleeding, to which he attributed their presence. It may be that the amounts he injected were insufficient to evoke the response. He did not inject or measure the high-density lipoproteins, our Fraction 4.

The histologic observations permit few conclusions. Chylomicrons probably do not enter the endocardium. One rat receiving chylomicrons showed lesions after a massive dose. At necropsy the serum showed significant amounts of lipoproteins of Fraction 2. In the intervening 5 hours this fraction may have been much higher. Although the injection of Fraction 4 was likewise followed by the appearance of lipoproteins of Fraction 2, no lesions were observed in these animals. Again it must be pointed out that analysis at the end of the 5-hour period gives no information about conditions existing during the interval. The appearance of lesions following the injection of Fraction 3 cannot absolutely be ascribed to the liproproteins of Fraction 3 for the same reason.

It is the lipoproteins of our Fractions 2 and 3 which the Donner group now implicate in the pathogenesis of human atherosclerosis.[15] Moreton,[16] on the other hand, believes that chylomicrons, our Fraction 1, are responsible for the disease. To our knowledge no one has as yet implicated the high-density lipoproteins, our Fraction 4. Barr[17] has even suggested that they may play a protective role.

SUMMARY

Human serum lipoproteins were separated into 4 fractions on the basis of density differences. The fractions were injected intravenously in rats. The rats were killed 5 hours later. The serum was analyzed for its lipid content and for its lipoprotein pattern. The hearts were examined microscopically for the presence of sudanophilic material within the endocardium.

Chylomicrons were removed very rapidly from the circulation. Higher density lipoproteins disappeared slowly.

The injection of one fraction was followed by the appearance of serum lipoproteins of other fractions. This phenomenon appeared in both density directions. The injection of low-density lipoproteins was followed by the appearance of certain higher density classes. This is thought to represent a normal pathway in the metabolism of low-density lipoproteins. The injection of high-density lipoproteins was followed by the appearance of those of lower density. This may be the result either of direct conversion of one class to another or of blockade of mechanisms involved in fat transport.

Focal deposits of sudanophilic material appeared within the endocardium of rats receiving the larger doses of all fractions except that of the highest density.

REFERENCES

1. Hirsch, E. F., and Weinhouse, S.: Role of Lipids in Atherosclerosis, Physiol. Rev. 23: 185, 1943.

2. Bragdon, J. H.: Transfusion Transfer of Experimental Atherosclerosis, Circulation 4: 466, 1951.
3. Bragdon, J. H.: Spontaneous Atherosclerosis in the Rabbit, Circulation 5: 641, 1952.
4. Bevans, M., Abell, L. L., and Kendall, F. E.: Production of Intimal Atherosclerosis by Intravenous Injection of Colloidal Cholesterol in Rabbits, Fed. Proc. 7: 269, 1948.
5. Bragdon, J. H., and Mickelsen, O.: Experimental Atherosclerosis in the Rat, Am. J. Path. 31: 965, 1955.
6. Page, I. H., and Brown, H. B.: Induced Hypercholesterolemia and Atherogenesis, Circulation 6: 681, 1952.
7. Bragdon, J. H., Havel, R. J., and Boyle, E.: Human Serum Lipoproteins. I. Chemical Composition of Four Fractions, J. Lab. & Clin. Med. 48: 36-42, 1956.
8. Havel, R. J., Eder, H. A., and Bragdon, J. H.: The Distribution and Chemical Composition of Ultracentrifugally Separated Lipoproteins in Human Serum, J. Clin. Invest. 34: 1345, 1955.
9. Ray, B. R., Davisson, E. O., and Crespi, H. L.: Experiments on the Degradation of Lipoproteins from Serum, J. Phys. Chem. 58: 841, 1954.
10. Pierce, F. T., Jr.: The Interconversion of Serum Lipoproteins *in vivo*, Metabolism 3: 142, 1954.
11. Jones, H. B., Gofman, J. W., Lindgren, F. T., Lyon, T. P., Graham, D. M., Strisower, B., and Nichols, A. V.: Lipoproteins in Atherosclerosis, Am. J. Med. 11: 358, 1951.
12. Graham, D. M., Lyon, T. P., Gofman, J. W., Jones, H. B., Yankley, A., Simonton, J., and White, S.: Blood Lipids and Human Atherosclerosis. II. The Influence of Heparin on Lipoprotein Metabolism, Circulation 4: 666, 1951.
13. Boyle, E., Bragdon, J. H., and Brown, R. K.: Role of Heparin in *in vitro* Production of Alpha$_1$ Lipoproteins in Human Plasma, Proc. Soc. Exper. Biol. & Med. 81: 475, 1952.
14. Bragdon, J. H., and Havel, R. J.: *In vivo* Effect of Anti-heparin Agents on Serum Lipids and Lipoproteins, Am. J. Physiol. 177: 128, 1954.
15. Gofman, J. W., Glazier, F., Tamplin, A., Strisower, B., and de Lalla, O.: Lipoproteins, Coronary Heart Disease, and Atherosclerosis, Physiol, Rev. 34: 589, 1954.
16. Moreton, J. R.: Chylomicronemia, Fat Tolerance, and Atherosclerosis, J. Lab. & Clin. Med. 35: 373, 1950.
17. Barr, D. P.: Some Chemical Factors in the Pathogenesis of Atherosclerosis, Circulation 8: 641, 1953.

REACTIVITY OF HEMOPHILIC PLASMA TO PLATELET THROMBOPLASTIN

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INTRODUCTION

NORMAL reactivity to thromboplastin of tissue origin and lack of reactivity to thromboplastin of platelet origin have been regarded as diagnostic features of hemophilic blood. (The term thromboplastin is used in the classic sense of a substance contained in tissue cells and platelets released from these elements in injury and having the property of initiating coagulation.) This lack of reactivity of hemophilic blood to platelet thromboplastin has been ascribed by numerous authors to deficiency of essential coagulation factors; it has now been reported that as many as four different factors may be involved, corresponding to different varieties of hemophilia.[1] On the other hand, Tocantins[2] has presented much evidence over a period of years that the coagulation defect of hemophilia is due to excess of an inhibitor, antithromboplastin. In 1951, Mann, Hurn, and Barker[3] reported that platelet thromboplastin undergoes a reaction with serum, resulting in markedly increased thromboplastic activity; this reaction, first observed with tissue thromboplastin, has been named the cothromboplastin reaction.[4, 5] It is generally recognized that addition of serum has little effect on the coagulation defect of plasma of classic hemophilia. However, it seemed worth-while to see whether hemophilic plasma might be reactive to platelet thromboplastin with which the cothromboplastin reaction had been completed.[6]

MATERIALS AND METHODS

The general plan of the experiments was essentially that previously reported.[3, 7] Platelets were separated from normal and from hemophilic plasma by differential centrifugation. After careful drawing off of the original plasma, the platelet sediment was redispersed and washed twice, each time with a volume of oxalated saline approximately equal to the original volume of plasma. Finally the platelets were redispersed in a volume of saline equal to 1/20 of the original volume of plasma. These platelet suspensions were allowed to react with equal volumes of M/40 $CaCl_2$ and a 1:5 dilution of serum for 12 minutes, after which the systems were tested for thromboplastin activity toward platelet-poor hemophilic plasma and also for thrombin activity with fibrinogen. In some experiments, thromboplastin activity in the two-stage system was also tested, with use of the method previously described.[8] Prothrombin utilization was also determined by a two-stage technique. The details and data of representative experiments are presented in the tables.

Five patients with classic hemophilia were studied. All of these showed a typical clinical picture, including hemarthroses, and had a history of hemophilia in maternal male

From the Palo Alto Hospital Research Foundation, Palo Alto, Calif.
Received for publication Nov. 14, 1955.

relatives. The clotting time of recalcified plasma and prothrombin utilization were restored to essentially normal by 1/10 volume of adsorbed plasma and were relatively little affected by a like amount of serum. (The plasma was adsorbed with 1/10 volume of M/10 suspension of calcium phosphate.) In addition, the platelets and plasma were studied in the case of a man whose clinical picture was also that of hemophilia but whose prothrombin utilization was virtually unaffected by adsorbed plasma and was restored by serum, thus meeting the criteria for PTC deficiency. Finally, similar tests were done on a woman showing the picture of PTA deficiency, namely a lifelong hemorrhagic diathesis with abnormal prothrombin utilization partially restored by both plasma and serum. It is of course realized that, since the specific plasmas from the original authors were lacking for mixing experiments, these patients could conceivably have some other hemophilia-like disease.

RESULTS

After normal platelet thromboplastin had undergone the cothromboplastin reaction it proved fully as reactive with hemophilic as with normal plasma, giving coagulation time of 7 to 13 seconds in all five cases studied (Tables I, II). (Table I gives preliminary data; the hemophilic plasma and platelets used for the illustrative experiments of Tables I to III are all from the same subject.) Such platelet thromboplastin after the cothromboplastin reaction also caused extremely rapid conversion of prothrombin to thrombin in hemophilic plasma in the two-stage system, complete conversion requiring only 15 seconds (Table III). No recognized source of antihemophilic factor was added in these experiments.

When instead of normal platelets, hemophilic platelets were used, very little thromboplastic activity resulted on reaction with normal serum (Tables II and IV). The shortest clotting time obtained was 40 seconds in one hemo-

TABLE I. REACTION OF HEMOPHILIC PLASMA WITH PLATELET THROMBOPLASTIN IN THE ONE-STAGE SYSTEM, WITHOUT PRELIMINARY REACTION

REACTION MIXTURE	CLOTTING TIME (SECONDS)
0.1 ml. Hemophilic plasma + 0.2 ml. M/80 $CaCl_2$	1,420
0.1 ml. (Hemophilic plasma + 1/10 vol. adsorbed plasma) + 0.2 ml. M/80 $CaCl_2$	240
0.1 ml. (Hemophilic plasma + 0.1 ml. normal platelets + 0.1 ml. (normal serum 1:5 in M/40 $CaCl_2$)	1,080

TABLE II. REACTION OF HEMOPHILIC PLASMA WITH PLATELET THROMBOPLASTIN IN THE ONE-STAGE SYSTEM, WITH PRELIMINARY COTHROMBOPLASTIN REACTION

PRELIMINARY REACTION* MIXTURE		FINAL REACTION MIXTURE	CLOTTING TIME (SECONDS)
0.2 ml. Normal platelets 0.2 ml. M/40 $CaCl_2$ 0.2 ml. Normal serum 1:5	12′	0.1 ml. Prelim. mixt. + 0.1 ml. M/40 $CaCl_2$ + 0.1 ml. Hemophilic plasma	11
		0.1 ml. Prelim. mixt. + 0.2 ml. 1% fibrinogen	1,140
0.2 ml. Hemophilic platelets 0.2 ml. M/40 $CaCl_2$ 0.2 ml. Normal serum 1:5	12′	0.1 ml. Prelim. mixt. + 0.1 ml. M/40 $CaCl_2$ + 0.1 ml. Hemophilic plasma	178
		0.1 ml. Prelim. mixt. + 0.2 ml. 1% fibrinogen	1,200

*After 12 minutes preliminary reaction, 0.1 ml. aliquots of the preliminary mixture tested in the final reaction mixture as noted.

TABLE III. REACTIÓN OF HEMOPHILIC PLASMA WITH PLATELET THROMBOPLASTIN IN THE TWO-STAGE SYSTEM

REACTION MIXTURE, IN ORDER OF ADDITION	REACTION INTERVAL (SECONDS)	CLOTTING TIME (SECONDS); 0.2 ML. REACT. MIXT. + 0.2 ML. FIBRINOGEN-ACACIA*
(0.2 ml. normal platelets + 0.2 ml. M /40 $CaCl_2$ + 0.2 ml. normal serum 1:5), 12 minutes	15	15
Then + 0.3 ml. buffer + 0.3 ml. hemophilic plasma 1:25	30	15
	60	17
0.2 ml. Normal platelets + 0.2 ml. M/40 $CaCl_2$ + 0.3 ml. buffer + 0.3 ml. hemophilic plasma 1:25 + 0.2 ml. normal serum 1:5	60	>60
	120	55
	240	19†

*Equal parts 1% fibrinogen and 12% gum acacia.[8]

†With the slower conversion rate when the preliminary reaction is omitted, the yield of thrombin is lower because of the relatively high antithrombin content of the system.

TABLE IV. REACTIVITY OF VARIOUS ABNORMAL PLASMAS TO THEIR OWN PLATELETS TREATED WITH NORMAL SERUM

DIAGNOSIS	CLOTTING TIME (SECONDS)
Hemophilia	40
Hemophilia	178
Hemophilia	91
Hemophilia	105
Hemophilia	180
PTC Deficiency	8
PTA Deficiency	12

Test procedure as in Table II.

philic; all others exceeded 100 seconds. Platelets from the cases of PTC and PTA deficiencies reacted like normal platelets. When hemophilic serum was allowed to react with normal platelets, it proved normally reactive, but PTC and PTA deficient sera were hypoactive.

The amounts of thrombin which formed in the preliminary mixtures were minute, far less than in most such experiments in the literature. Such quantities of thrombin alone will not clot adsorbed plasma, with its high content of antithrombin.

DISCUSSION

The experiments show that hemophilic plasma reacts very rapidly with platelets which have undergone the cothromboplastin reaction, without addition of any recognized source of antihemophilic factor. It is not claimed that these experiments were conducted in the total absence of such a factor; indeed it is hard to say just what sort of evidence could indicate the total absence of this or any other coagulation factor. The amount of washing of the platelets was the same that has been employed by most workers in the field, e.g., Biggs and Douglas,[9] and is known to be adequate to remove all plasma constituents not actually combined with the platelets, e.g., prothrombin.[10] If, however, antihemophilic factor is actually associated with or adsorbed upon the platelets, it might not be possible to remove it completely, at least without greatly impairing platelet activity. This is known to be true for the labile prothrombin conversion factor.[10, 11] In this case it would appear that such a strongly associated active substance should be considered part of the platelet from a

functional point of view. In the foregoing experiments, after identical treatment, hemophilic platelets reacted very differently than did normal platelets.

SUMMARY

Normal platelet thromboplastin which has undergone the cothromboplastin reaction with dilute serum is highly reactive toward hemophilic plasma. Hemophilic platelets do not acquire such activity by reaction with dilute serum.

REFERENCES

1. Spaet, T. H., Aggeler, P. M., and Kinsell, B. G.: A Possible Fourth Plasma Thromboplastin Component, J. Clin. Invest. **33:** 1095-1102, 1954.
2. Tocantins, L. M., and Carroll, R. T.: Separation and Assay of a Lipid Antithromboplastin From Human Brain, Blood, Plasma, and Plasma Fractions. Blood Clotting and Allied Problems. Transactions of the Second Conference, New York, 1949, Josiah Macy, Jr., Foundation, pp. 11-28.
3. Mann, F. D., Hurn, M., and Barker, N. W.: Platelets and the Coagulation Defect Caused by Dicumarol, Am. J. Clin. Path. **21:** 814-819, 1951.
4. Mann, F. D.: Cothromboplastin Assay, Am. J. Clin. Path. **19:** 861-864, 1949.
5. Mann, F. D., and Hurn, M.: The Complex Mechanisms of the Quick Prothrombin Test and the Effect of Dicumarol, Am. J. Clin. Path. **20:** 225-232, 1950.
6. Mann, F. D., and Hurn, M. M.: Reactivity of Hemophilic Plasma to Platelet Thromboplastin, Fed. Proc. **14:** 413, 1955.
7. Mann, F. D., and Hurn, M.: Intermediate Reactions in Blood Coagulation, Am. J. Physiol. **175:** 65-68, 1953.
8. Hurn, M., and Mann, F. D.: The Determination of Prothrombin (Two Stage Method) and Antithrombin Using Commercially Available Reagents, Am. J. Clin. Path. **17:** 741-746, 1947.
9. Biggs, R., and Douglas, A. S.: The Thromboplastin Generation Test, J. Clin. Path. **6:** 23-29, 1953.
10. Hjort, P., Rapaport, S. I., and Owren, P. A.: Evidence That Platelet Accelerator (Platelet Factor 1) Is Adsorbed Plasma Proaccelerin, Blood **10:** 1139-1150, 1955.
11. Mann, F. D., Hurn, M., and Magath, T. B.: Observations on the Conversion of Prothrombin to Thrombin, Proc. Soc. Exper. Biol. & Med. **66:** 33-35, 1947.

TOXICITY OF SCHIZOPHRENICS' BLOOD SERUM IN TISSUE CULTURE

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NUMEROUS authors have claimed that humoral changes are involved, directly or indirectly, in the etiology of schizophrenia. Reiter,[1] in 1937, reasoned that if the blood of schizophrenic patients contains toxic substances, then replacement of their blood with that of healthy persons should result in detoxification and hence mental improvement. Replacement transfusions which he performed on four schizophrenic patients brought about a remarkable, though transitory, improvement in three of the patients. Reiter noted that the idea was far from new as an attempt had been made to cure a patient of mental disease by a transfusion of calf's blood, apparently with some success, in 1667.

Buscaino[2] reported that certain abnormal substances occur in the urine of patients having schizophrenia, involutional melancholia, some types of extrapyramidal syndromes, or high fever. He maintained that when silver nitrate was added to urine, a black precipitate formed, in contrast to a white precipitate, or one of intermediate color, in other cases. He believed the reaction to be specific for the diseases mentioned and to be of diagnostic value. However, Bettzieche,[3] and later Müller,[4] demonstrated the reaction to be unspecific and dependent on the relationship between uric acid and chlorides in the urine.

Katzenelbogen[5] made chemical analyses of the blood and of the cerebrospinal fluid of schizophrenic patients. He was unable to find any constant differences from normal values, but he did notice that "out of 20 patients, 17 showed deviations from the accepted normal limits for one or another constituent, either in blood or cerebrospinal fluid or both."

Tscherkes and Mangubi[6] noted that the growth index of the roots of *Lupinus albus* was smaller when the roots were grown in schizophrenic blood serum than when grown in the blood serum of healthy individuals. Macht,[7] who had originally developed this phytopharmacologic method, later confirmed these results and also showed that the cerebrospinal fluid from schizophrenic patients was as toxic as their blood serum.

Gamper, Kral, and Stein[8] observed the production of inflammatory exudate following the injection of schizophrenic cerebrospinal fluid into the

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In cooperation with the Saskatchewan Committee on Schizophrenic Research, supported by the Department of National Health and Welfare, Canada.
Presented in part at the meeting of the American Association of Anatomists, April, 1955.
Received for publication Nov. 25, 1955.

anterior chambers of the eyes of rabbits. Gamper and Kral reported that cerebrospinal fluid[9] and urine[10] of schizophrenic patients was toxic when injected subcutaneously into white mice. Reiter,[1] who was unable to confirm the latter observations, suggested that tissue cultures might be more sensitive test material. However, A. Fischer, who performed the tests on osteoblast cultures for him, failed to demonstrate toxic effects of the cerebrospinal fluid of schizophrenic patients.

Lazell and Prince[11] reported that the blood serum of patients having schizophrenia, diluted 1:1,000, killed the tadpoles of *Rana catesbiana* in three days or less; whereas tadpoles lived in the blood serum of normal individuals, diluted 1:1,000, for the duration of the experiment, that is, for almost one month. Similar experiments by R. Fischer[12] showed that schizophrenic blood serum was toxic to the larvae of *Xenopus levis,* but that during sudden weather changes the toxicity of the serum decreased.

On the assumption that tissue culture techniques would likely provide more sensitive methods for the detection of toxicity in schizophrenic blood, I began work on this problem, at the time unaware that A. Fischer, on the suggestion of Reiter,[1] had already used tissue culture techniques for similar purposes.

METHODS AND MATERIALS

Cells strains L and HeLa were chosen for the testing of schizophrenic blood. Both strains grow on glass surfaces. This is an advantage because blood plasma, which is otherwise needed to support cells in tissue cultures, may be omitted.

Strain L cells which were developed in the laboratory of Earle[13, 14] were used. This strain was derived from the subcutaneous tissue of a normal C_3H mouse. After the strain had been made malignant by treatment with 20-methylcholanthrene, a single cell was isolated, and from it a clone cell strain was developed. A culture of this strain was obtained from Earle in 1953, and the strain has been successfully maintained in this laboratory since then.

The strain HeLa was developed in the laboratory of Gey[15, 16] from an epidermoid carcinoma of the cervix uteri. A culture of this strain has been grown continuously in this laboratory since 1954.

Stock cultures of strain L cells are routinely cultured in eight-ounce Blake bottles at 37° C. The medium consists of 4 parts horse serum, 6 parts Hanks' balanced salt solution,[17] and and 0.5 part embryo extract. The embryo extract is routinely prepared from homogenized 9-day-old chick embryos. The homogenate is diluted 1:1 with Hanks' balanced salt solution. After centrifuging twice, the supernatant fluid is stored at −20° C. until it is used.

Strain HeLa cells are grown in Leighton tubes at 37° C., in a medium consisting of 3 parts pooled human blood serum and 7 parts Hanks' balanced salt solution.

The toxicity tests were carried out in the following way:

1. Cells were scraped from the walls of the stock bottles and suspended in the medium. The number of cells in the suspension was determined by placing a drop of the suspension in a hemocytometer, counting the cells, and calculating the number of cells per milliliter.

2. Approximately 150,000 cells, suspended in the medium, were put into each of a series of Leighton culture tubes.

3. The cultures were incubated at 37° C. for 3 to 4 days. When a growth of healthy cells was obtained, the old medium was removed and the cells were washed with Hanks' balanced salt solution.

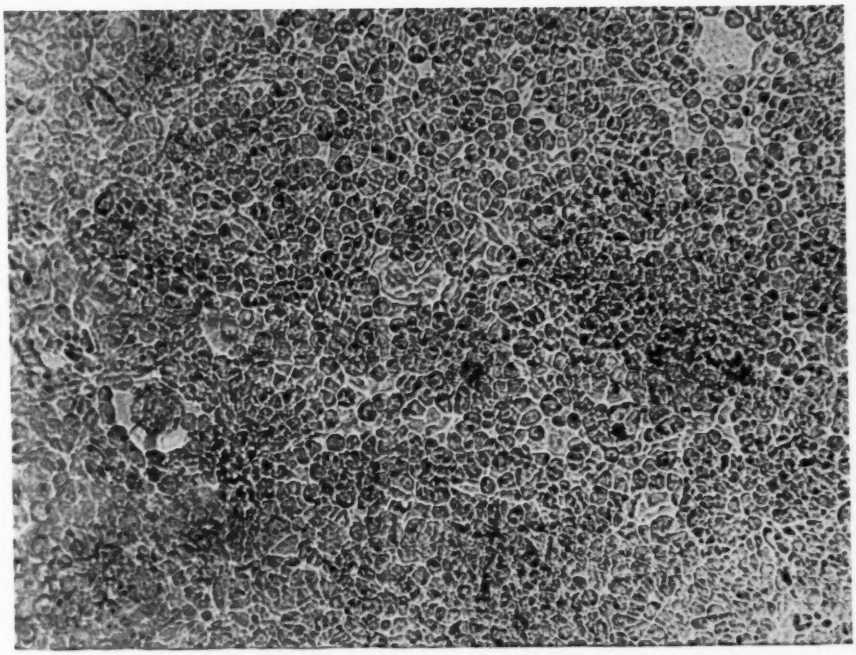

Fig. 1.—Strain L cells, 48 hours after addition of blood serum from young healthy adult. (×126.)

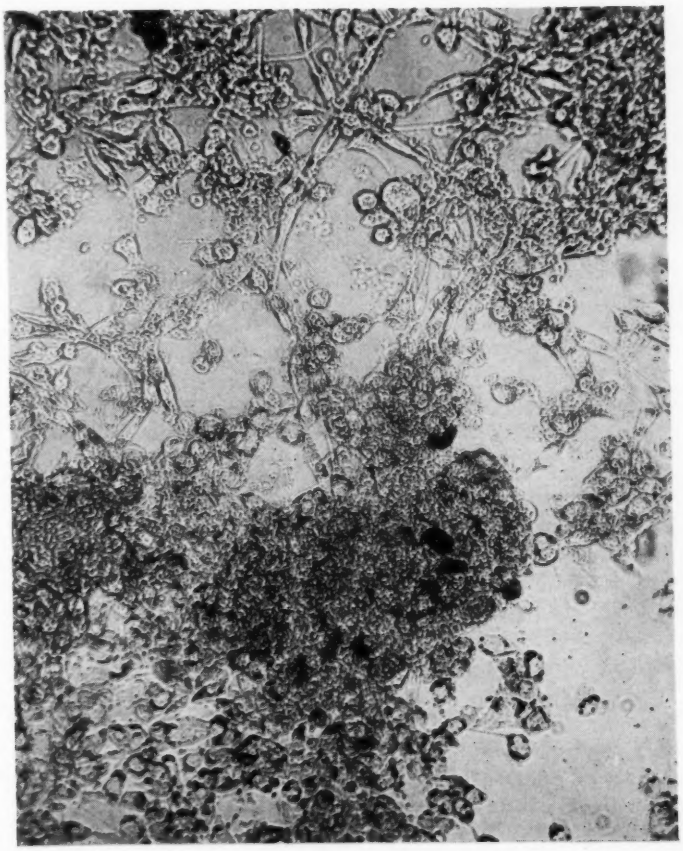

Fig. 2.—Strain L cells, 48 hours after addition of blood serum from hebephreniac. (Case 33, Saskatchewan Hospital, North Battleford.) (×126.)

4. The test medium, i.e., the blood serum to be tested diluted 1:1 with Hanks' balanced salt solution, was added. Usually 3 to 4 tubes were used for each serum sample.

The serums were obtained from blood samples collected before breakfast, with use of sterile, scrupulously clean needles and syringes. The blood was transferred to clean, sterile tubes having nontoxic rubber stoppers.[18] Special care was taken to exclude patients who had recently received medication and persons who had complicating conditions such as infections and menstruation.

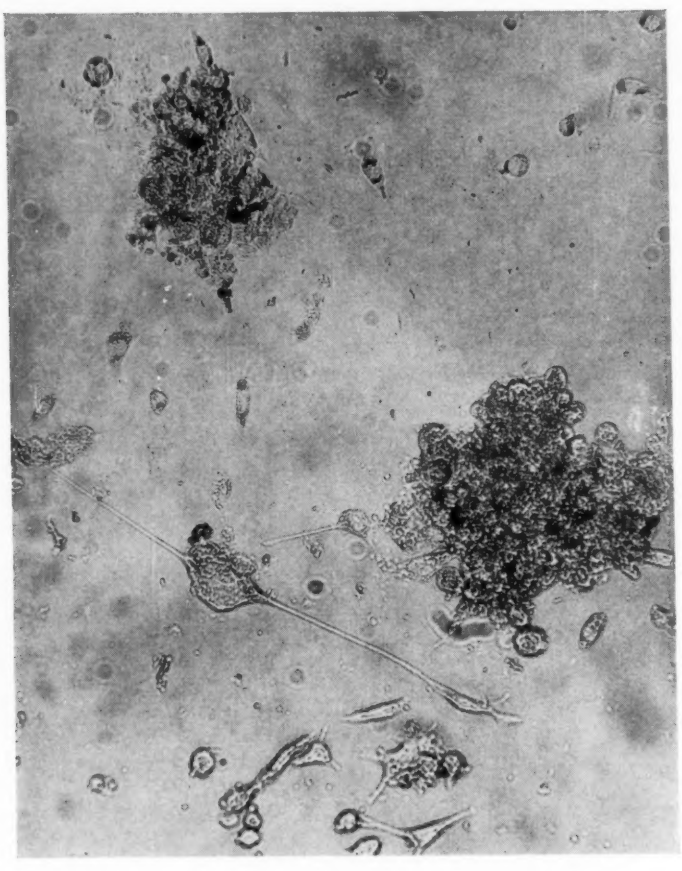

Fig. 3.—Strain L cells, 48 hours after addition of blood serum from paranoid schizophrenic. (Case 24, Saskatchewan Hospital, North Battleford.) (×126.)

Blood from schizophrenic patients was collected at the Saskatchewan Hospital at North Battleford, at the Munroe Wing of the Regina General Hospital, Regina, and at the University Hospital, Saskatoon. For comparative purposes blood samples were also obtained from medical students and staff members of the College of Medicine, University of Saskatchewan, and from staff members at the Munroe Wing, Regina. Blood samples from healthy and schizophrenic persons in Regina and from schizophrenic patients in North Battleford were kept chilled but not frozen by packing them in large thermos bottles containing ice, and were shipped immediately to Saskatoon. On arrival, usually not more than 24 hours after shipping, the blood was centrifuged, and the serum was removed and stored at 4° C. until used.

In order that the conditions of the material shipped to Saskatoon might be paralleled, blood samples obtained locally were also kept chilled for a comparable time before the serum was removed and stored at 4° C.

The samples were identified by numbers only, until the tests were completed. Altogether, serum samples from 109 individuals were tested, of which 29 were healthy persons and 80 were diagnosed by psychiatrists as having schizophrenia.

During the test period of 3 to 4 days, the cultures were observed microscopically each day. Toxic serum produced cell degeneration and cell necrosis (Figs. 1, 2, and 3). In the cultures one could see the cells rounding up, increasing in granularity, detaching themselves from the glass surface, becoming transparent, and losing their cell outlines. One could also see cell debris, abnormal cell forms (mainly large cells with long thin processes), and clusters in which neither cell boundaries nor nuclei could be distinguished. The extent of these changes provided the basis for grading the toxicity of the various serum samples. The degrees of toxicity were designated as follows: zero (0), when no toxic effect could be detected; 1 plus (+), when only a few rounded-up, degenerating cells were seen; 2 plus (++), when in most microscopic fields, healthy cells predominated over degenerating cells; 3 plus (+++), when there were more degenerating cells than healthy cells; and 4 plus (++++), when all but a few scattered cells were dead. At the termination of the test, the average of the daily toxicity grades of the serums were calculated. These average grades were considered to be the toxicity grades of the serum samples.

Generally, the most severe effects of toxic serum were observed during the first 24 hours. If the damage to the cells during this time was not too severe, the condition of the culture was somewhat improved by the end of a week. The effects were different if the initial cultures were too heavily populated. In such cases, besides cell necrosis and degeneration, large clumps of cells appeared within 24 hours after the addition of the medium containing toxic serum. Apparently the cells in the clumps had a better chance of survival because outgrowths of healthy cells emerged from the clumps within a few days. This condition complicated the grading of the cultures.

Four serum samples which showed high toxicity were heated for 35 minutes in an oven at 56° C., then retested for toxicity.

RESULTS

While samples of blood serum from 80 schizophrenic patients showed all degrees of toxicity when tested on strain L cells, 74 per cent of these samples were toxic to a degree of 3 plus to 4 plus, and 26 per cent were not toxic or were toxic to a degree of 1 plus to 2 plus (Table I). Of the serum samples from 29 healthy persons, only 10 per cent showed toxicities of 3 plus to 4 plus, whereas 90 per cent were not toxic or were toxic to a degree of 1 plus to 2 plus (Table I). According to the Chi square test the two groups differed to a highly significant degree as to their toxicity in cultures of strain L cells.

The blood serums of 8 schizophrenic patients were retested after several months. In 6 cases the toxicities were still within the same range and in two cases the toxicity was decreased (Table II).

TABLE I. EFFECT OF BLOOD SERUM FROM SCHIZOPHRENIC PATIENTS AND FROM HEALTHY PERSONS ON STRAIN L CELL CULTURES

	TOTAL NUMBER OF CASES	DEGREE OF SERUM TOXICITY				
		0	+	++	+++	++++
Number of schizophrenic patients	80	7	4	10	19	40
Number of healthy individuals	29	5	13	8	1	2

TABLE II. CONSTANCY OF DEGREE OF TOXICITY OF SCHIZOPHRENIC BLOOD SERUM TO STRAIN L CELL CULTURES OVER A PERIOD OF SEVERAL MONTHS

CASE NO.	CLINICAL DIAGNOSIS	DEGREE OF BLOOD SERM TOXICITY		NO. OF MONTHS BETWEEN 1ST AND 2ND TEST
		AT 1ST TEST	AT 2ND TEST	
87 (34)	Schizophrenia (Catatonia)	0	0	4
88 (35)	Schizophrenia (Hebephrenia)	+++	++++	4
86 (33)	Schizophrenia (Hebephrenia)	+++	++++	5
91 (44)	Schizophrenia (Paranoid)	+++	+++	5
85 (23)	Schizophrenia (Undifferentiated)	++++	++++	6
89 (11)	Schizophrenia (Paranoid)	++++	++	7
90 (13)	Schizophrenia (Paranoid)	++++	++++	7
53 (36)	Schizophrenia (Hebephrenia)	++	0	9

Ten samples of serum which were toxic to strain L cells were not toxic to strain HeLa cells. Thus, since there appeared to be no difference in the effects of normal and schizophrenic serum on strain HeLa cells, further testing on this strain was discontinued.

Heating of toxic blood serum at 56° C. for 35 minutes resulted in its complete detoxification.

DISCUSSION

The results of these experiments indicate that in most cases schizophrenic blood serum differs from the blood serum of healthy persons in its effect on strain L cells. This observation suggests several questions:

1. In what way does the serum of schizophrenics exert its toxic effect on the cells?

2. How specific is the toxicity to the schizophrenic state?

3. If toxicity is observed in serum of nonschizophrenics, is the cause of the toxicity identical with that causing toxicity in schizophrenic serum?

These questions, unanswered at present, are being investigated further in this laboratory.

Referring to the third question, two or more entirely different toxins, in appropriate concentrations, could bring about the same degree of destruction to cells, although each might attack the cells in a different manner. The serum of two apparently healthy individuals was toxic to the degree of 4 plus (Table I). On further investigation it was found that one serum sample had been obtained from a female student who had been menstruating at the time. Previously, Macht and Lubin[19] had reported that the blood serum of menstruating women contains a toxic substance which inhibits the growth of *Lupinus albus* seedlings. The second serum sample having a toxicity of 4 plus was obtained from a male student who had fainted while the blood was being withdrawn. The two cases suggest that a stress reaction may play a part in bringing about serum toxicity. If such be the case, then the problem would arise of differentiating toxicity due to stress from that occurring in the serum during the schizophrenic state. It is possible, however, that the latter toxicity is also related to a stress reaction.

Some differences between A. Fischer's experiments on osteoblast cultures and these experiments should be mentioned. First, he used cerebrospinal fluid from schizophrenic patients, whereas we used blood serum; second, the concentrations he used may have been too low to produce toxic effects, since I observed no toxic effect when serum was present in concentrations of 10 per cent or less; third, it is probable that he used a plasma coagulum as a support for the cells of his cultures. There is evidence that plasma coagulum is able, to some extent, to protect cells from toxins, whereas cells grown directly on glass are more vulnerable.

As mentioned, schizophrenic serum was toxic to strain L cells but not to strain HeLa cells. It is difficult to explain this observation at present. However, one has to keep in mind that these strains differ as to derivation: strain L is derived from connective tissue and strain HeLa from epithelial tissue. There is also a species difference: strain L cells were explanted from a mouse and strain HeLa cells from a human. Finally, strain L cells are adapted to grow in horse serum and HeLa cells in human serum. It is possible that because of the latter difference, strain L cells are more sensitive to the slightest changes in human serum than strain HeLa cells, which are accustomed to a medium containing human serum.

Because of the difference in the effect of toxic serum on the two cell strains and because of the noxious effect of toxic serum on strain L cells, one must also consider the possibility that a virus may be involved. So far, however, the presence of a virus in the blood serum of schizophrenics has not been demonstrated.

Obviously, much further work will be necessary to establish whether or not a specific substance or substances can be identified which are responsible for the toxicity of the serum of schizophrenics and whether or not a virus can be definitely ruled out.

SUMMARY

Blood serum of schizophrenic patients was more toxic to strain L cells in tissue cultures than serum of healthy individuals. The toxicity disappeared after heating the serum at 56° C. for 35 minutes. It has not yet been established how specific the toxicity is to schizophrenia nor how the toxic serum acts on the cells.

The blood serum of schizophrenic persons was not toxic to strain HeLa cells.

I wish to thank Dr. A. Hoffer and Dr. A. F. Meszaros for blood specimens from patients and for clinical information about the patients. The technical assistance of Miss Frances Madigan is greatly appreciated.

REFERENCES

1. Reiter, P. J.: Untersuchungen zur Beleuchtung der Intoxikationstheorie bei der Dementia praecox mit besonderer Berücksichtigung der Versuche mit Totaltransfusionen, Ztschr. f. d. ges. Neurol. Psychiat. **160:** 598-614, 1937-38.
2. Buscaino, V. M., quoted by Katzenelbogen, S.: The Busciano Black Reaction in Urine. Its Genesis and Diagnostic Value With Special Reference to Mental Diseases, Am. J. Psychiat. **8:** 1021-1043, 1929.

3. Bettzieche, F.: Chemische und klinische Untersuchungen über die Schwarzreaction von Buscaino in Harn von Gesunden und Kranken, Deutsche med. Wchnschr. **53**: 1511-1512, 1927.
4. Müller, A.: Zur Chemie der Busciano-Therapie, Schweiz. med. Wchnschr. **58**: 379-383, 1928.
5. Katzenelbogen, S.: Studies on Schizophrenia; Chemical Analyses of Blood and Cerebrospinal Fluid, Arch. Neurol. & Psychiat. **37**: 881-894, 1937.
6. Tscherkes, L. A., and Mangubi, M. I.: Phytotoxishe Eigenschaften des Blutes bei Schizophrenie, Ztschr. f. d. ges. Neurol. u. Psychiat. **132**: 815-820, 1931.
7. Macht, D. I.: Pharmacologic Reactions of Normal and Psychotic Blood Sera, South. M. J. **43**: 1049-1057, 1950.
8. Gamper, E., Kral, A., and Stein, R.: Untersuchungen über die Wirkung von pathologischem Liquor cerebrospinalis bei Einbringung in die Vorderkammer des Kaninchenauges, Ztschr. f. d. ges. Neurol. u. Psychiat. **141**: 689-701, 1932.
9. Gamper, E., and Kral, A.: Experimentell-biologische Untersuchungen zum Schizophrenieproblem, Ztschr. f. d. ges. Neurol. u. Psychiat. **146**: 567-598, 1933.
10. Gamper, E., and Kral, A.: Weitere experimentell-biologische Untersuchungen zum Schizophrenieproblem, Ztschr. f. d. ges. Neurol. u. Psychiat. **150**: 252-271, 1934.
11. Lazell, E. W., and Prince, L. H.: The Study of Causative Factors of Dementia Praecox, U. S. Vet. Bur. M. Bull. **5**: 40-41, 1929.
12. Fischer, R.: Stress and the Toxicity of Schizophrenic Serum, Science **118**: 409-410, 1953.
13. Earle, W. R.: Production of Malignancy in Vitro. IV. The Mouse Fibroblast Cultures and Changes Seen in the Living Cells, J. Nat. Cancer Inst. **4**: 165-212, 1943.
14. Sanford, K. K., Earle, W. R., and Likely, J. D.: The Growth in Vitro of Single Isolated Tissue Cells, J. Nat. Cancer Inst. **9**: 229-246, 1948-49.
15. Gey, G. O., Caffman, W. D., and Kubicek, M. T.: Tissue Culture Studies of the Proliferative Capacity of Cervical Carcinoma and Normal Epithelium, Cancer Res. **12**: 264, 1952.
16. Scherer, W. F., Syverton, J. T., and Gey, G. O.: Studies on the Propagation in Vitro of Poliomyelitis Viruses, J. Exper. Med. **97**: 695-710, 1953.
17. Hanks, J. H.: *in* An Introduction to Cell and Tissue Culture, Minneapolis, 1955, Burgess Publishing Company.
18. Parker, R. C., Morgan, J. F., and Morton, H. J.: Toxicity of Rubber Stoppers for Tissue Cultures, Proc. Soc. Exper. Biol. & Med. **76**: 444-445, 1951.
19. Macht, D. I., and Lubin, D.: A Phytopharmacological Study of Menstrual Toxin, J. Pharm. and Exper. Therap. **22**: 413-466, 1924.

ESTIMATION OF TOTAL BODY CHLORIDE IN MAN BY RADIOBROMIDE DILUTION

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SEVERAL investigators have demonstrated the similarity of bromide and chloride distribution in animals, including man.[1-9] In general, bromide enters tissues in proportion to the amount of chloride present, the only important exceptions being the brain, spinal fluid, and gastric juice[4, 5, 8-10]; thus the use of bromide to give an estimation of body chloride has been a feasible procedure. A refinement of this is the use of radioactive bromide.[2, 4, 7, 11] Bromide and radiobromide are of utility for this purpose since there is no chloride isotope of appropriate half-life available for such dilution studies in man. The studies reported here supply data on the use of radiobromide in the estimation of total body chloride in man.

METHODS

Fifteen medical students, eleven men and four women, were used in this study. The subjects were carefully selected for good general health, the absence of medication, and no evidence of metabolic abnormalities; and they were cautioned against excess salt consumption, exercise, or sweating. Nine children were used to study spinal fluid distribution rates. The procedure for the adults consisted of: (1) intravenous injection of radiobromide solution, (2) a wait from one to forty-eight hours for radiobromide distribution, (3) urine collection over the waiting period, (4) plasma sampling of the subjects at the end of the waiting period, (5) assay of samples, and (6) calculation of the total body chloride from radiobromide dilution.

Radiobromide^{82} was obtained from Oak Ridge National Laboratories and prepared as a sterile solution of NaBr in physiologic NaCl. The injection of the radiobromide solution was done with use of a three-way stopcock with a syringe and a reservoir, according to the method described by Barnett and Fellers.[12] In the adults, from 1 to 4 ml. of physiologic saline containing 1 to 2 μc of Br^{82} per kilogram of body weight was injected into an antecubital vein of one arm; the injection apparatus was then rinsed into the vein with an equal volume of saline three times. From 5 to 10 ml. of blood was withdrawn into a heparinized syringe from the opposite arm at the end of each waiting period. The plasma was immediately separated from the cells. Plasma chloride was determined by the method of Van Slyke and Hiller.[13] Radioactivity of the plasma and urine was determined with use of a mica-windowed bell Geiger counter tube. The samples, 1 ml. of plasma or 2 ml. of urine, were dried in a weighed aluminum planchet into which a drop of colloidal graphite had been added.[14] Duplicate counts were made on each sample, each measurement to a minimum of 10,000 counts. Control samples of a dilution of the injection fluid were run

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Received for publication Dec. 23, 1955.
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at the same time. Counts were corrected for background, resolving time, and decay (half-life of Br^{82} – 35.5 hours). Because of the low penetrating power of Br^{82} beta-rays (0.465 mev.), an absorption correction for the solid residue in the planchet was made. This was determined empirically, and the measured values were in agreement with values theoretically determined according to the method of Reid.[15] The corrections used are shown in Fig. 1.

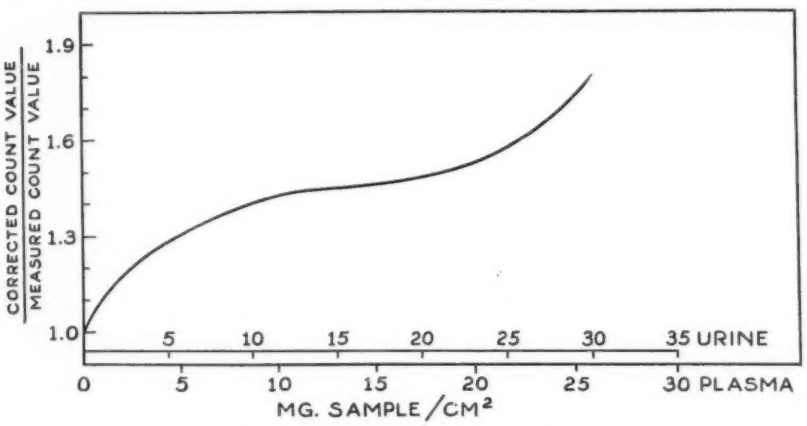

Fig. 1.—Correction factor for self-absorption of Br^{82} radiation from dried plasma and urine samples.

Total body chloride was computed as follows[2]:

$$\text{Total body Cl} = \text{Plasma Cl} \left[\frac{Br^{82}\text{ administered} - Br^{82}\text{ excreted}}{\text{Plasma } Br^{82}}\right]$$

In the spinal fluid experiments after injection of 1 to 2 μc Br^{82} per kilogram of body weight, a period of from 3 to 24 hours was allowed to elapse before sampling. In addition to the blood sample, a specimen of the spinal fluid was collected by lumbar puncture and was analyzed for chloride and Br^{82} by the methods used for plasma. Nonradioactive plasma was used as a spinal fluid diluent for the counting to avoid scattering errors occasioned by low sample weights.

The radiation hazard in the use of Br^{82} in this manner is inconsequential if ordinary precautions are observed. Total body radiation to the subject is under 0.8 equivalent roentgens, and no tissue except gastric juice develops an appreciably higher concentration of Br^{82} than the average concentration.

RESULTS AND DISCUSSION

Table I contains data from the measurements on eight men after various Br^{82} equilibration times of from 1 to 48 hours. In each of the four subjects in which sampling was done prior to 24 hours, there is good agreement between the 5-hour and the 24-hour determinations. In the other four subjects, correspondence was not as good between the 24-hour and 48-hour determinations. With this as a basis, a 24-hour equilibration time was used for the succeeding observations. Table II contains data from the measurements after 24-hour equilibration on the fifteen adults, four with determinations repeated after intervals of about 60 days. Total body chloride ranged from 24.7 to 37.6 meq. per kilogram of body weight and averaged 32.1 meq. per kilogram with a standard deviation of 11.8 per cent for the men; 25.1 to 28.1 meq. per kilogram of body

TABLE I. TOTAL BODY CHLORIDE DETERMINATIONS ON NORMAL ADULT MALES: 1 TO 48 HOURS AFTER Br^{82} INJECTION

SUBJECT	WT. (KG.)	SAMPLING TIME (HOURS)	% Br^{82} EXCRETED (CUMULATIVE)	A RETAINED Br^{82} Br^{82} PER LITER PLASMA	B MEQ.CL PER LITER PLASMA	[A] [B] TOTAL CHLORIDE (MEQ.)	MEQ.CL PER KG. BODY WEIGHT
C. B.	77.5	1	1.34	21.38	112.1	2,400	31.0
		2	1.87	22.71	110.1	2,500	32.3
		3	2.42	24.87	107.2	2,662	34.4
		5	3.34	24.17	111.5	2,696	34.8
		24	8.06	24.03	110.3	2,649	34.2
F. S.	73.6	1	0.177	22.90	115.2	2,640	35.6
		2	0.70	24.80	108.7	2,690	36.8
		3	1.41	27.37	111.3	3,044	41.3
		5	3.09	26.53	103.9	2,776	37.4
		24	6.77	26.33	103.5	2,777	37.7
R. H.	76.8	1	0.741	23.00	101.9	2,343	30.5
		2	0.741	24.17	113.9	2,754	35.9
		3	1.50	24.72	108.9	2,691	35.0
		5	2.40	24.43	103.9	2,538	33.0
		24	14.20	23.70	106.6	2,526	32.9
L. R.	75.0	1	0.323	23.41	116.1	2,718	36.2
		2	0.769	22.33	108.8	2,432	32.4
		3	1.21	23.90	110.0	2,629	35.0
		5	1.91	24.68	108.9	2,688	35.8
		24	6.78	22.84	116.1	2,653	35.4
A. S.	72.8	24	6.79	23.18	101.6	2,352	32.3
		48	13.43	19.46	101.4	1,974	26.3
H. B.	88.74	24	5.23	20.52	107.0	2,196	24.7
		48	10.43	19.46	108.0	1,958	22.1
J. A.	79.2	24	6.23	20.33	106.0	2,155	27.2
		48	10.78	18.50	105.1	1,946	24.5
C. B.	88.74	24	6.22	18.03	109.0	1,966	25.4
		48	13.30	17.95	112.1	2,012	26.0

weight and averaged 26.3 meq. per kilogram with a standard deviation of 5.3 per cent for the women. The combined group averaged 30.9 meq. per kilogram with a standard deviation of 13.6 per cent. Dunning and co-workers[6] reported in 52 normal individuals (26 men and 26 women) a mean total chloride of 31.6 ± 3.8 meq. per kilogram for males, 28.6 ± 3.3. meq. per kilogram for females, with a combined mean of 30.1 ± 3.9 meq. per kilogram as measured by the stable bromide-dilution technique. Ikkos and associates[11] reported in 21 adults, 12 men and 9 women, a mean exchangeable chloride of 29.5 ± 3.3 meq. per kilogram as measured by 20- to 24-hour Br^{82} dilution. These results are within experimental variation of the results reported in this paper. Gamble and co-workers[4] compared Cl^{38} with stable bromide spaces in five chronically ill human subjects and found that, compared to the Cl^{38} space, stable bromide space was 7 per cent higher. In another group of ten chronically ill patients they found the Br^{82} space to be 2 per cent higher than the Cl^{38} space. Assuming a three-fold concentration of Br^{82} in gastric juice containing 0.1 per cent of the body chloride, the total chloride calculated by the Br^{82} dilution formula would be 0.2 per cent too high.

TABLE II. TOTAL BODY CHLORIDE OF FIFTEEN ADULTS 24 HOURS AFTER Br^{82} INJECTION

	SUBJECT	WT. (KG.)	HT. (CM.)	% Br^{82} EXCRETED	A RETAINED Br^{82} Br^{82} PER LITER PLASMA	B MEQ.CL PER LITER PLASMA	[A] [B] TOTAL CHLORIDE (MEQ.)	MEQ. CL PER KG. BODY WEIGHT
MEN:	C. B.	77.5	176	8.06	24.03	110.3	2,649	34.2
	*C. B.	77.5	176	6.22	18.03	109.0	1,966	25.4
	F. S.	73.6	189	6.77	26.33	103.5	2,777	37.7
	R. H.	76.8	182	14.2	23.70	106.6	2,526	32.9
	L. R.	75.0	173	6.78	22.84	116.1	2,653	35.4
	J. G.	67.7	191	7.01	22.03	102.1	2,249	33.2
	S. G.	64.8	168	3.11	20.19	102.6	2,072	32.0
	A. S.	72.8	175	6.79	23.18	101.6	2,352	32.3
	*A. S.	71.4	175	4.47	21.18	105.5	2,229	35.8
	H. B.	88.7	176	5.31	25.47	105.5	2,688	30.3
	*H. B.	88.7	176	5.23	20.52	107.0	2,196	24.7
	E. L.	78.7	178	4.75	24.05	104.2	2,506	32.9
	J. A.	79.2	179	5.64	25.38	103.9	2,637	33.3
	*J. A.	79.2	179	6.23	20.33	106.0	2,155	27.2
	M. B.	73.2	185	5.03	25.31	101.4	2,560	35.0
								Av. = 32.1 ± 3.8
WOMEN:	L. K.	69.1	173	8.77	16.07	107.9	1,733	25.1
	V. S.	44.8	154	11.7	11.82	106.9	1,263	28.2
	M. B.	62.0	172	8.44	15.78	104.6	1,649	26.6
	P. W.	56.9	167	9.92	13.44	107.2	1,441	25.3
								Av. = 26.3 ± 1.4
								Composite Av. (M. and W.). = 30.9 ± 4.2

*Determinations on same subject at a later date.

TABLE III. DISTRIBUTION OF Br^{82} INTO CEREBRAL SPINAL FLUID

SUBJECT	TIME AFTER INJECTION (HRS.)	A* CSF Br^{82} PLASMA Br^{82}	B* CSF CL PLASMA CL	$\frac{A}{B}$
D. C.	3	0.245	1.19	0.205
D. A.	6	0.422	1.18	0.357
L. D.	12	0.420	1.16	0.346
R. W.	12	0.319	1.17	0.272
J. H.	12	0.503	1.15	0.437
R. A.	24	0.336	1.17	0.287
J. T.	24	0.751	1.17	0.642
L. E. D.	24	0.345	1.13	0.308
S. H.	24	0.350	1.13	0.310

*Concentrations are calculated on the volumes of wet tissue.

Another source of error is the difference in Br^{82}-to-chloride ratio in the spinal fluid as compared to plasma water. Table III contains the data from observations on nine children in which the rate of entrance of Br^{82} from the plasma into the spinal fluid was studied. Even after 24 hours, the Br^{82} has not approached the distribution of chloride in the spinal fluid. Wallace and Brodie[10] found that the chloride-to-administered bromide ratio in spinal fluid never approached that of plasma even after continuous administration over a period of eight days. Weir and Hastings[5] reported a similar finding but did not specify the time intervals. Gamble and associates[4] reported a brain tumor patient study in which spinal fluid Br^{82} and Cl^{38} reached 30 per cent and 46 per cent, respectively, of the plasma concentration three hours after administration. Mason[8] reported in a study of five patients after prolonged bromide ad-

ministration that the spinal fluid contained a lower bromide-chloride ratio than serum. Zetterholm[17] found a blood-to-spinal fluid bromide ratio after prolonged bromide administration to 50 subjects to average 3.2. It appears, therefore, that there is a rather rapid accumulation of bromide to about 20 to 30 per cent of its relative plasma abundance followed by a relatively slow accrual of more bromide. It is assumed by Wallace and Brodie and by Weir and Hastings that other central nervous system tissues have relatively the same chloride-to-bromide ratio as the spinal fluid. Assuming the total nervous system chloride to be 2.1 per cent of the total chloride[18] and assuming 40 per cent Br^{82} equilibration in nervous tissue after 24 hours, the total body chloride calculated by the Br^{82} dilution formula would be 1.3 per cent too low. The combination of gastric juice and spinal fluid corrections would indicate a correction of 1 per cent should be added to the body chloride calculated by the formula.

Measurement of plasma Br^{82} dilution 24 hours after injection appears to offer a satisfactory method for the estimation of total body chloride. The addition of a 1 per cent correction to the calculated value to compensate for gastric juice and spinal fluid Br^{82}-chloride distribution differences is a refinement which may be used by optimistic investigators, but the small size of this correction is in itself supportive evidence of the validity of the Br^{82} dilution method.

SUMMARY

Total body content of chloride has been estimated in a series of fifteen medical students by the method of radiobromide dilution.

Results obtained by this method compare favorably with those reported by other investigators using other techniques.

Total body chloride is approximately 32 meq. per kilogram in young men and about 26 meq. per kilogram in young women.

Evaluation of known possible sources of appreciable error using this technique, namely, variation in spinal fluid and gastric juice, produces a correction factor of 1 per cent, which may be added to the calculated total chloride.

REFERENCES

1. Deane, N., Ziff, M., and Smith, H.: The Distribution of Total Body Chloride in Man, J. Clin. Invest. **31**: 200-203, 1952.
2. Forbes, G. B., Reid, A., Bondurant, J., and Etheridge, J.: Estimation of Total Body Chloride in Young Infants by Radiobromide Dilution, Proc. Soc. Exper. Biol. & Med. **83**: 871-872, 1953.
3. Wallace, G. B., and Brodie, B. B.: The Distribution of Administered Bromide in Comparison With Chloride and Its Relation to Body Fluids, J. Pharmacol. & Exper. Therap. **65**: 214-219, 1939.
4. Gamble, J. L., Jr., Robertson, J. W., Hannigan, C. A., Foster, C. G., and Farr, L. E.: Chloride, Bromide, Sodium and Sucrose Spaces in Man, J. Clin. Invest. **32**: 483-489, 1953.
5. Weir, E. G., and Hastings, A. B.: The Distribution of Bromide and Chloride in Tissues and Body Fluids, J. Biol. Chem. **129**: 547-558, 1939.
6. Dunning, M. F., Steele, J. M., and Berger, E. Y.: Measurement of Total Body Chloride, Proc. Soc. Exper. Biol. & Med. **77**: 854-858, 1951.
7. Gamble, J. L., Jr., and Robertson, J. S.: Volume of Distribution of Radioactive Chloride in Dogs; Comparisons With Sodium, Bromide and Inulin Spaces, Am. J. Physiol. **171**: 659-667, 1952.
8. Mason, M. F.: Halide Distribution in Body Fluids in Chronic Bromide Intoxication, J. Biol. Chem. **113**: 61-74, 1936.

9. Patrick, S. I., and Eadie, G. S.: Bromides in Spinal Fluid and Serum, Am. J. Physiol. **168:** 254-259, 1952.
10. Wallace, G. B., and Brodie, B. B.: The Distribution of Iodide, Thiocyanate, Bromide and Chloride in the Central Nervous System and Spinal Fluid, J. Pharmacol. Exper. Therap. **65:** 220-226, 1939.
11. Ikkos, D., Ljunggren, H., Luft, R., and Sjogren, B.: Content and Distribution of Potassium and Chloride in Adults, Metabolism **4:** 231-237, 1955.
12. Barnett, H. L., and Fellers, F. X.: A Simple Quantitative Method for Intravenous Injection of Small Volumes of Fluid, Science **106:** 401-402, 1948.
13. Van Slyke, D. D., and Hiller, A.: Application of Sendroys Iodometric Chloride Titration to Protein Containing Fluids, J. Biol. Chem. **167:** 107-124, 1947.
14. Reid, A. F., and Robbins, M. C.: Colloidal Graphite in the Preparation of Samples for Gas-flow Counting, Science **116:** 148-149, 1952.
15. Reid, A. F.: Detection and Measurement of Radioactive Tracers. Preparation and Measurement of Isotopic Tracers, Ann Arbor, 1946, Edwards Brothers.
16. Davenport, H. W., and Fisher, R. B.: Mechanism of the Secretion of Acid by the Gastric Mucosa, Am. J. Physiol. **131:** 165-175, 1940.
17. Zetterholm, S.: Blood-Spinal Fluid Permeability to Bromide in Closed Head Injuries, Acta Psychiatrica et Neurologica Supplement **45:** 1-139, 1947.
18. Shohl, A. T.: Mineral Metabolism, New York, 1939, Reinhold Publishing Corp.

EFFECTS OF INSULIN HYPOGLYCEMIA ON RENAL HEMODYNAMICS

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THE frequent occurrence of renal disease and of insulin hypoglycemia in the clinical course of patients with diabetes mellitus makes the effects of insulin hypoglycemia on renal hemodynamics of clinical interest. Accordingly, measurements of the effective renal plasma flow and the glomerular filtration rate before, during, and after insulin hypoglycemia were undertaken in three groups of people: normal subjects, patients with diabetes mellitus but without clinical evidence of renal disease, and patients with renal disease.

The results indicate that hypoglycemia severe enough to produce symptoms is associated with reductions in the effective renal plasma flow and in the glomerular filtration rate and with an increase in the filtration fraction.

MATERIALS

The normal subjects studied were patients in the hospital, with comparatively minor illnesses. Four diabetic patients were selected who would have prominent hyperglycemia before insulin was administered. These subjects did not demonstrate evidence of renal disease by history, urinalysis, or phenolsulfonphthalein excretion test.

The four patients with renal disease that were studied had no urea retention. This group included three patients with the nephrotic syndrome and one patient with intercapillary glomerulosclerosis demonstrated by needle biopsy of the kidney.

Although no sedation was used, a number of the subjects slept during part of the procedure, and two subjects (E. B. and J. B.) slept throughout the study. If any subject selected for study seemed to become apprehensive, the study was discontinued and the clearances were not determined.

METHODS

Standard techniques for inulin and sodium para-aminohippurate clearances were used.[1, 2] The subjects were fasted for 12 hours, prehydrated, and maintained in a hydrated state. Each urinary collection was terminated by washing the bladder with air and physiologic saline. Arterial pressures were determined by direct measurement through the arterial needle or in some subjects with a sphygmomanometer.

No collections were begun until at least 20 minutes after the last manipulation of the patient. Three collections of urine were made of at least 14 minutes each in the control period with the arterial blood samples obtained at the midpoint of each collection. The subject was then given insulin (hyperglycemic-factor-free) in a dosage of 0.1 to 0.25 units per kilogram of body weight intravenously for the nondiabetic subjects and 15 to 30 units intravenously for the patients with diabetes. Urinary and blood collections were continued through the hypoglycemic period and during a period after the hypoglycemia had been terminated by glucose intravenously. Symptomatic hypoglycemia, when it occurred,

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This work was supported (in part) by United States Public Health Fellowship HF 4809 and (in part) by the Life Insurance Medical Research Fund.
Received for publication Dec. 27, 1955.
*Research Fellow of the U. S. Public Health Service, 1954-55.

was allowed to continue for 20 to 30 minutes before it was terminated with glucose. The onset of symptomatic hypoglycemia could be clearly determined clinically by the appearance of sweating and weakness. Profuse sweating alone was used as the identifying criterion in 2 subjects (E. B. and J. B.) who were asleep during the hypoglycemic period.

The blood samples for inulin and sodium para-aminohippurate determinations were immediately centrifuged and a plasma filtrate was made by the cadmium sulfate method of Fujita and Iwatake.[3] Inulin was determined by the resorcinol method as modified by Roe.[4] Sodium para-aminohippurate determinations were carried out with use of the method of Selkurt[2] with the N-naphthyl ethylenediamine dihydrochloride recrystallized with hydrochloric acid, as described by Bratton.[5] The Somogyi method[6] as modified by Nelson[7] was used for the blood glucose determinations.

In two subjects the sodium para-aminohippurate extraction was determined by renal vein catheterization. In the remaining subjects an extraction of 90 per cent was assumed.[8]

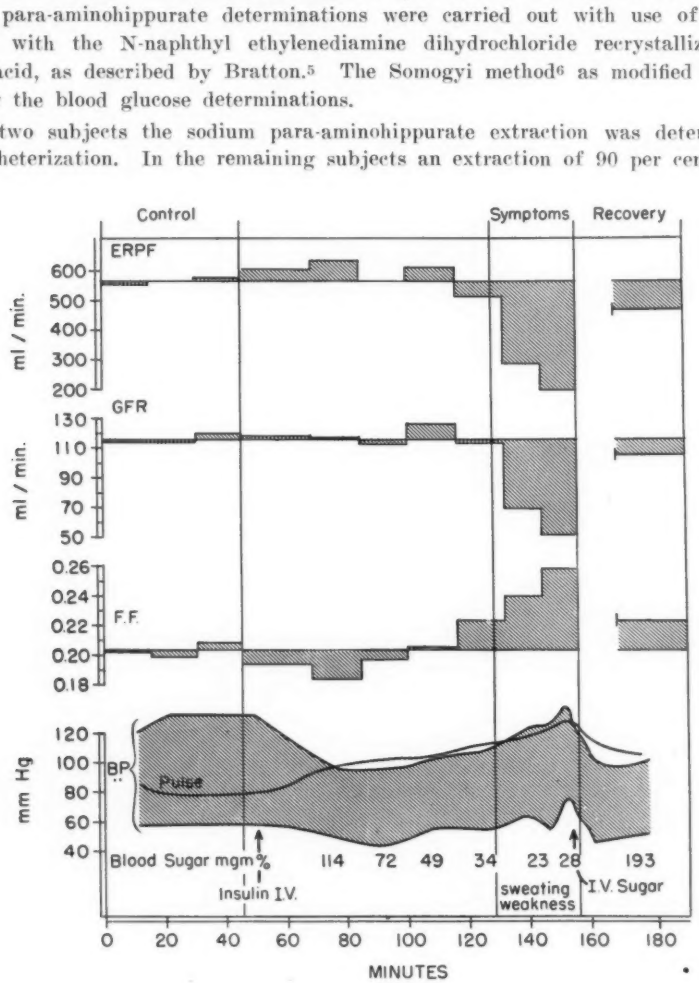

Fig. 1.—Diagram showing the characteristic response in renal hemodynamics to symptomatic insulin hypoglycemia (Patient J. S.).

RESULTS

The normal subjects studied were readily divided into two groups: those who had symptomatic hypoglycemia, and those who had no hypoglycemia or who had hypoglycemia without symptoms or evident signs of it. Table I, *A* gives the data for the ten normal subjects who developed symptoms of hypoglycemia, each demonstrating a decrease in the effective renal plasma flow and

glomerular filtration rate and an increase in the filtration fraction. The typical response of a subject (J. S.) to symptomatic insulin hypoglycemia is illustrated in Fig. 1.

Fig. 2 presents the average of the changes found in these same ten subjects in the hypoglycemic and recovery periods as compared to the control periods. The data for each subject are expressed in percentages of the mean value for the control periods for the same subject. Demonstrable also in Fig. 2 is the rebound seen in the recovery period with the measurements returning toward or even beyond the control values.

Subjects who had hypoglycemia without weakness and sweating did not demonstrate the renal hemodynamic changes found in the subjects who developed symptomatic hypoglycemic reactions. Table I, *B* presents the data for four subjects who developed asymptomatic hypoglycemia. In this group, the levels of blood glucose differed little from the levels that occurred in the subjects recorded in Table I, *A*.

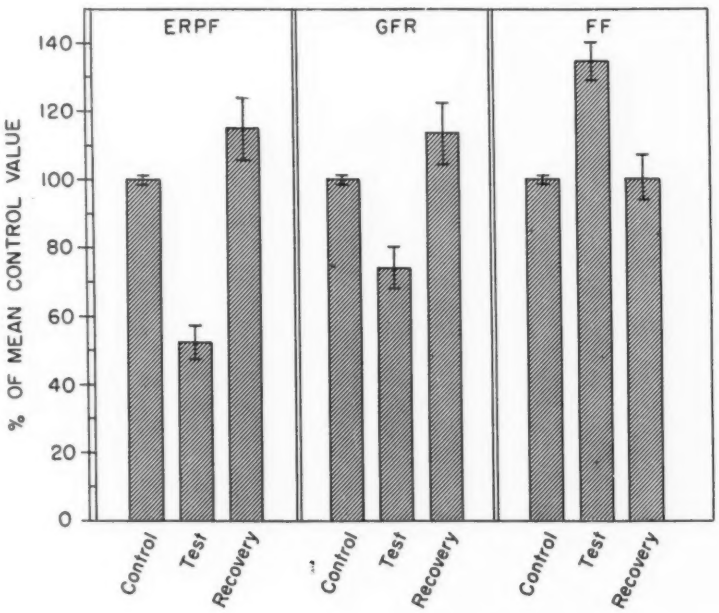

Fig. 2.—Graph showing the mean percentile changes in effective renal plasma flow, glomerular filtration rate, and filtration fraction in the subjects with symptomatic insulin hypoglycemia.

In each of the four patients with diabetes mellitus, the blood glucose level at the onset of the study was high, and the decrease in the blood glucose level after insulin was pronounced but not productive of symptoms. No decrease in effective renal plasma flow or glomerular filtration rate occurred in this group (Table I, *C*). It is interesting to note that though these four patients had no clinical evidence of renal disease, the renal clearances in two were abnormal.

TABLE

	NAME	EFFECTIVE RENAL PLASMA FLOW ML./MIN.* CONTROL PERIOD	HYPOGLYCEMIC PERIOD	DIFFERENCE	GLOMERULAR FILTRATION RATE ML./MIN.* CONTROL PERIOD	HYPOGLYCEMIC PERIOD	DIFFERENCE
A. Hypoglycemia with symptoms (10 normal subjects)	L. S.	610	366	−244	98	91	− 7
	J. M.	1,081	320	−761	160	69	−91
	J. S.	680	234	−446	137	60	−77
	L. M.	728	422	−306	112	80	−32
	M. O.	822	514	−308	142	121	−21
	A. S.	524	312	−212	111	90	−21
	R. W.	799	507	−292	123	88	−35
	R. H.	489	376	−113	101	93	− 8
	J. B.	666	312	−354	144	103	−41
	E. B.	1,042†	560	−482	126†	108	−18
	Mean	744	392	−352	125	90	−35
	S. E.	62	33	57	6	6	9
	p			<0.001			<0.01
B. Hypoglycemia without symptoms (3 normal subjects, one subject with glomerulonephritis)	E. S.	669	706	+ 37	151	156	+ 5
	J. J.	802	833	+ 31	142	152	+10
	L. P.	542	610	+ 68	115	131	+16
	S. B.	554	657	+103	47	55	+ 8
	Mean	642	702	+ 60	114	124	+10
C. Large fall in blood sugar without symptoms	J. R.	443	528†	+ 85	72	79†	+ 7
	J. S.	681	718	+ 35	137	140	+ 3
	A. P.	393	412	+ 19	96	86	−10
	M. M.	575	721	+146	106	124	+18
	Mean	523	595	+ 72	103	107	+ 4
D. Hypoglycemic reactions in patients with renal disease (S. W. had intercapillary glomerulosclerosis; J. F., J. C., and A. A. had nephrotic syndrome)	S. W.	494	227	−267	40	26	−14
	J. F.	527	306	−221	87	51	−36
	J. C.	181	97	− 84	26	20	− 6
	A. A.	454	240	−214	79	59	−20
	Mean	414	218	−196	58	39	−19
	Per cent of control		53			69	

*Corrected to 1.73 sq. M. body surface area.

†Designates average of only two collection periods. All other control periods and asymptomatic hypoglycemic periods are the averages of three collection periods.

The four patients with renal disease who had hypoglycemic symptoms showed a decrease in the effective renal plasma flow and glomerular filtration rate, with the percentile changes similar to the percentile changes in the normal subjects with symptomatic hypoglycemia (Table I, *D*).

In the normal subjects, the rate of excretion of urine during the control periods was 9.85 ± 0.69* ml. per minute, during the hypoglycemic periods 1.93 ± 0.50 ml. per minute, and in the recovery periods 1.95 ± 0.63 ml. per minute.

DISCUSSION

A comparison of the findings in regard to renal hemodynamics in the various groups of persons studied indicates clearly that the changes are related neither to the absolute blood glucose concentration during hypoglycemia nor to the degree or rapidity of fall in blood glucose. The sleep pattern

*All ± values represent the standard error.

I

FILTRATION FRACTION			BLOOD GLUCOSE MG. PER CENT			BLOOD PRESSURE MM. Hg MEAN SYSTOLIC/MEAN DIASTOLIC			
CONTROL PERIOD	HYPOGLYCEMIC PERIOD	DIFFERENCE	CONTROL	MINIMAL	DIFFERENCE	CONTROL PERIOD	HYPOGLYCEMIC PERIOD	DIFFERENCE SYST.	DIFFERENCE DIAST.
0.161	0.249	+0.088	79	42	− 37	--	--	--	--
0.149	0.217	+0.068	73	23	− 50	143/80	150/79	+ 7	− 1
0.202	0.258	+0.056	--	23	--	127/60	127/63	0	+ 3
0.154	0.190	+0.036	84	49	− 35	122/68	126/67	+ 4	− 1
0.173	0.236	+0.063	82	46	− 36	130/67	129/58	− 1	− 9
0.200	0.288	+0.088	82	45	− 37	--	--	--	--
0.153	0.173	+0.020	80	39	− 41	149/89	138/80	−11	− 9
0.207	0.246	+0.039	81	37	− 44	147/84	151/78	+ 4	− 6
0.216	0.328	+0.112	94	24	− 70	142/81	176/58	+34	−23
0.121†	0.193	+0.072	71	41	− 30	112/74	116/67	+ 4	− 7
0.174	0.238	+0.064	80	37	− 42	134/75	139/68	+ 5	− 7
0.010	0.015	0.009	4	3	4	5/ 4	7/ 3	5	3
		<0.001			<0.001			>0.3	>0.05
0.226	0.221	−0.005	82	50	− 32	121/66	125/60	+ 4	− 6
0.177	0.182	+0.005	85	49	− 36	--	--	--	--
0.212	0.215	+0.003	75	36	− 39	119/60	114/48	− 5	−12
0.085	0.084	−0.001	85	51	− 34	138/67	140/63	+ 1	− 4
0.175	0.176	+0.0005	82	46	− 36	126/64	126/57	0	− 7
0.162	0.150†	−0.012	220	88	−132	110/55	112/52	+ 2	− 3
0.201	0.195	−0.006	180	49	−131	130/64	124/60	− 6	− 4
0.244	0.209	−0.035	196	95	−101	134/78	132/81	− 2	+ 3
0.184	0.172	−0.012	252	92	−160	169/82	174/83	+ 5	+ 1
0.198	0.182	−0.016	212	81	−131	138/70	138/69	0	− 1
0.082	0.115	+0.033	113	36	− 77	148/84	153/78	+ 5	−6
0.165	0.166	+0.001	69	34	− 35	126/66	137/74	+11	+ 8
0.141	0.207	+0.066	82	45	− 37	--	--	--	--
0.174	0.246	+0.072	57	39	− 18	132/74	136/72	+ 4	− 2
0.141	0.184	+0.043	80	38	− 42	135/75	142/75	+ 7	0

in those subjects who slept did not correlate with the periods of the described changes. The only positive correlation which is apparent is the coincidence of the changes in the renal hemodynamics with the occurrence of symptoms of hypoglycemia.

The decrease in the effective renal plasma flow with a lesser decrease in the glomerular filtration rate, and thus an increase in the filtration fraction, is typical of the pattern of changes that occurs as a result of renal efferent arteriolar vaso-constriction.[9] This type of response is also seen in shock,[10-12] in fright,[13] and following epinephrine administration.[14-16]

The blood pressures during the control and hypoglycemic periods are presented in Table I. The minor variations that occurred would not appear to represent hypotensive reactions. The occurrence of the renal hemodynamic changes in the two subjects who slept through the study supports the belief that fright was not the causative factor.

Boothby and Wilder,[17] in 1923, were the first investigators to note similarities between the clinical syndrome seen in people receiving epinephrine and that seen in people suffering hypoglycemic reactions, thus posing the possi-

bility of epinephrine activity causing certain symptoms of hypoglycemia. The pattern of the response in renal hemodynamics to hypoglycemia, the presence of sweating, and the frequent increase in pulse pressure seen in the subjects with symptomatic hypoglycemia are compatible with an increase in epinephrine activity. Epinephrine blocking agents were used in further studies to evaluate the role of epinephrine, but the results were too variable for reliable evaluation.

Symptomatic hypoglycemia was associated with the same degree of reduction in effective renal plasma flow and glomerular filtration rate in patients with renal disease as was found in the normal subjects. These alterations were fleeting during the course of this acute study, but the possibility cannot be excluded that more prolonged and severe hypoglycemia might have deleterious effects in diabetics with renal disease.

The findings in this study were not in agreement with those reported by May,[18] whose abstract, in 1950, did not relate a decrease in para-aminohippurate clearance in psychotic patients during insulin therapy, but there was mention of a rise in clearance after termination of hypoglycemia in some subjects.

Antidiuresis was observed 45 to 60 minutes after the administration of insulin. This occurred even though the water intake had been constant, and irrespective of the presence or absence of hypoglycemia. This antidiuresis in response to insulin was reported by Miller and Bogdonoff,[19] who also found that hypoglycemia was not necessary for the effect. That a collection error was not a factor in causing the clearance changes is evidenced by the change which occurred in the filtration fraction, the lack of a decrease in clearances in the asymptomatic hypoglycemic subjects, and the return to normal clearances after the termination of the hypoglycemia even though the antidiuresis was still prominent.

SUMMARY

Consistent changes occur in renal hemodynamics in subjects with symptomatic hypoglycemic reactions. A decrease in the effective renal plasma flow, a decrease in the glomerular filtration rate, and an increase in the filtration fraction are found. It is proposed that these changes are a result of epinephrine activity secondary to the hypoglycemia.

I wish to express my gratitude for the advice and assistance of Dr. J. D. Myers and for the technical assistance of Mrs. Rita Lang and Misses Dorothy Frederick and Charie Belland.

REFERENCES

1. Goldring, W., Chasis, H., Ranges, H. A., and Smith, H. W.: Relations of Effective Renal Blood Flow and Glomerular Filtration to Tubular Excretory Mass in Normal Man, J. Clin. Invest. **19**: 739-750, 1940.
2. Selkurt, E. C.: Measurement of Renal Blood Flow, Meth. Med. Res. **1**: 196-199, 1949.
3. Fujita, A., and Iwatake, D.: Determination of Blood Sugar Without Enzymes, Biochem. Ztschr. **242**: 43-60, 1931.
4. Roe, J. H., Epstein, J. B., and Goldstein, N. P.: A Photometric Method for the Determination of Inulin in Plasma and Urine, J. Biol. Chem. **178**: 839-845, 1949.
5. Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Agent for Sulfanilamide Determination, J. Biol. Chem. **128**: 537-550, 1939.
6. Somogyi, M.: Determination of Blood Sugar, J. Biol. Chem. **160**: 69-73, 1945.
7. Nelson, N.: A Photometric Adaptation of the Somogyi Method for the Determination of Glucose, J. Biol. Chem. **153**: 375-380, 1944.

8. Warren, J. V., Brannon, E. S., and Merrill, A. J.: A Method of Obtaining Renal Venous Blood in Unanesthetized Persons With Observations on the Extraction of Oxygen and Sodium Para-aminohippurate, Science, **100**: 108-110, 1944.
9. Smith, H. W.: The Kidney, New York, 1951, Oxford University Press.
10. Van Slyke, D. D.: The Effects of Shock on the Kidney, Ann. Int. Med. **28**: 701-722, 1948.
11. Corcoran, A. C., and Page, I. H.: Effects of Hypotension Due to Hemorrhage and of Blood Transfusions on Renal Function in Dogs, J. Exper. Med. **78**: 205-224, 1943.
12. Lauson, H. D., Bradley, S. E., and Cournand, A.: The Renal Circulation in Shock, J. Clin. Invest. **23**: 381-402, 1944.
13. Smith, H. W.: The Physiology of the Renal Circulation, Harvey Lect. **35**: 166-222, 1939-1940.
14. Berne, R. M., Hoffman, W. K., Kagan, A., and Levy, M. N.: Response of the Normal and Denervated Kidney to L'epinephrine and L'norepinephrine, Am. J. Physiol. **171**: 564-571, 1952.
15. Richards, A. N., and Plant, O. H.: Action of Adrenalin on Kidney, Am. J. Physiol. **59**: 184-190, 1922.
16. Richards, A. N., and Plant, O. H.: Action of Minute Doses of Adrenalin and Pituitrin on the Kidney, Am. J. Physiol. **59**: 191-202, 1922.
17. Boothby, W. M., and Wilder, R. M.: Preliminary Report on the Effect of Insulin on the Rate of Heat Production and Its Significance in Regard to the Calorigenic Action of Adrenalin, Med. Clin. N. Am. **7**: 53-56, 1923.
18. May, L. G., Bennett, A., Schoomer, M., and Gregory, R. L.: The Effects of Insulin Hypoglycemia Upon Inulin and Para-aminohippurate Clearances, J. Lab. & Clin. Med. **36**: 964, 1950.
19. Miller, J. H., and Bogdonoff, M. D.: Antidiuresis Associated With Administration of Insulin, J. Appl. Physiol. **6**: 509-512, 1954.

THERAPEUTIC EFFECT OF HYALURONIDASE IN EXPERIMENTAL TUBULAR NECROSIS OF KIDNEY

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ACUTE renal failure in rats was produced by a modification of Koletsky's method.[1] Morphologic study of kidneys at varying time intervals after a period of total ischemia has shown that acute tubular necrosis is characterized by disintegration of tubule cells with precipitation of a homogeneous eosinophilic material throughout the length of the renal tubule. Simultaneously with the regrowth of tubular epithelium the intratubular deposits disappear. Serial morphologic follow-up gave the impression that the disappearance of the intratubular deposits proceeded by means of enzymatic dissolution and not by a "flushing" of particulate debris down the length of the nephron. Experiments suggested themselves which would decide whether or not in animals with experimental renal disease the postulated intratubular enzymatic debridement could be accelerated by the administration of suitable enzyme preparations.

METHODS

Adult male rats of the Wistar strain, average weight 250 grams, were anesthetized with intraperitoneal injection of 6 mg. pentobarbital sodium. The left kidney was excised and its pedicle ligated. The right renal pedicle was clamped for periods of 90 to 95 minutes. Pedicle clamps were made of bent steel wire; their jaws exerting a pressure of about 300 Gm. The jaws of the clamps were coated with 2 mm. outside diameter red-rubber catheter tubing. At the end of the clamping period, the kidneys were replaced in the abdominal cavity and the incision was sutured. Control animals received one injection of 0.5 ml. saline intraperitoneally the evening after the operation and two daily injections of 0.5 ml. saline for 4 days thereafter or else for the duration of their postoperative life. Experimental animals received the test substance dissolved in 0.5 ml. saline following the same schedule.

RESULTS

Controls.—Each series of experimental agents was run with an equal number of simultaneous controls. Thirty-one of 44 control rats died between the second and the fifth day after the operation, a mortality rate of 71 per cent. Only those animals which died between the second and the fifth day after the operation or which survived indefinitely were counted. Deaths on the day of operation or the first day thereafter were excluded from the data be-

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This study was supported in part by Grant H-1158 from the National Heart Institute, United States Public Health Service.
Received for publication Dec. 30, 1955.

cause they were interpreted as resulting from surgical interference and not from uremia. The typical lesions of acute tubular necrosis were found in the kidneys of the dead animals (Table I).

*Plasmin**.—Eight rats received 5 mg. of a purified plasmin preparation with each injection. The mortality in this series was 100 per cent.

Plasmin Plus Hyaluronidase.—Six rats received 5 mg. plasmin and 75 turbidity reducing units (TRU) hyaluronidase (Wydase)† with each injection. The mortality in this series was 100 per cent.

Hyaluronidase.—Forty-nine rats received 150 TRU hyaluronidase (Wydase) with each injection. Thirty-five of these animals survived indefinitely, a mortality of 28 per cent. There are 2.5 chances in 10,000 that the difference in control mortality and in hyaluronidase mortality is a phenomenon caused by random variation.

TABLE I. EFFECTS OF VARIOUS ENZYME PREPARATIONS ON RATS WITH EXPERIMENTAL RENAL FAILURE

AGENT	NUMBER OF RATS	DAY OF DEATH POST OPERATION					SURVIVORS
		1	2	3	4	5	
None (controls)	44 (+4)	3	1	14	10	7	13
Plasmin	6 (+2)	1	1	4	1	1	0
Plasmin-hyaluronidase	6			3	1	1	0
Hyaluronidase	49 (+4)	2	2	6	5	3	35

+ denotes the number of animals which died on the day of operation or the first preoperative day, presumably from causes connected with the operative trauma and not from uremia.

DISCUSSION

Our serial morphologic studies and also parallel investigations by Brun,[2] Daniel and associates,[4] and Casey and Woolf[3] have disclosed that in animals as in man the initial stage of the acute renal failure syndrome is characterized by the deposition of homogeneous, pink-staining material throughout the length of the renal tubule, especially in its proximal convoluted segment. This eosinophilic deposit tends to disappear as tubular regeneration progresses; the intratubular cylinders decrease in diameter as if dissolved by enzymes which act on their perimeter.

The chemical constitution of intratubular precipitates is not definitely established, but it is logical to assume that intratubular deposits are of a material which is similar to urinary casts and that this material originates to a large extent from the blood plasma and is conveyed to the tubule by glomerular filtration. The proteinaceous nature of the urinary casts and of intratubular precipitates has always been taken for granted. It is, however, a fact that the normal blood plasma contains mucoprotein in the alpha- and beta-globulin fractions which filter through the glomerular membrane (Sellers and associates[7]), that up to 80 per cent of the nondialyzable material in urine consists of mucoid material (Hamerman and associates[8]) and that the urinary casts are insoluble in saline (Addis[5]) and contain metachromatic material (Oliver[6]). The facts mentioned above make it very likely that the precipitates which obstruct the nephron contain a mucoprotein component. While the

*Plasmin obtained through the courtesy of R. Smith, M.D., Sharp & Dohme, Inc.
†Wydase was obtained through the courtesy of G. E. Farrar, M.D., Wyeth Laboratories.

kidneys are known to contain several types of proteolytic enzymes, renal mucolytic enzymes have not been demonstrated, and it appears possible that the ability of the kidney to dispose of intratubular mucoprotein deposits is one of the limiting factors in its recovery from various types of injury. It is not definitely known whether or not hyaluronidase can hydrolyze or depolymerize one or more of the mucoproteins and glycoproteins which occur in plasma and in urine. If the therapeutic effect of large parenteral doses of hyaluronidase in experimentally induced acute tubular necrosis is due to an action of this enzyme on the mucoprotein deposits within the lumen of the nephron, it would be expected to reduce that degree of renal dysfunction which is caused by obstruction of the nephrons by intratubular deposits. However, the hyaluronidase effect could also be mediated by a reduction of interstitial edema or by a nonspecific extrarenal mechanism; the hyaluronidase effect cannot be explained on the basis of the results reported here.

SUMMARY

Unilateral nephrectomy was performed in 93 rats which survived more than one day after the operation. The remaining kidney was subjected to total ischemia for 90 to 95 minutes. Seventy-one per cent mortality was observed in 44 controls which received up to 4.5 ml. saline intraperitoneally in divided doses during four and one-half days. The mortality was 28.5 per cent in 49 rats in which hyaluronidase (Wydase), 600 TRU per kilogram of body weight was added to each injection. The mechanism of this therapeutic effect is not apparent. The increased survival of the hyaluronidase-treated rats is statistically highly significant.

REFERENCES

1. Koletsky, S.: Effects of Temporary Interruption of Renal Circulation in Rats, AMA Arch. Path. **58**: 592-603, 1954.
2. Brun, Claus: Acute Anuria, Copenhagen, 1954, Munksgaard.
3. Casey, W. C., and Woolf, W.: Needle Biopsy of the Kidneys, California Med. **83**: 353-359, 1955.
4. Daniel, P. M., Prichard, M. M. L., and Ward-McQuaid, J. N.: The Renal Circulation After Temporary Occlusion of the Renal Artery, Brit. J. Urol. **26**: 118-126, 1954.
5. Addis, T.: The Renal Lesion in Bright's Disease, Harvey Lectures **23**: 222-250, 1928.
6. Oliver, J.: New Directions in Renal Morphology, Harvey Lect. **40**: 102-155, 1944-1945.
7. Sellers, A. L., Roberts, S., Rask, I., Smith, S., III, Marmoston, J., and Goodman, H. C.: An Electrophoretic Study of Urinary Protein in the Rat, J. Exper. Med. **95**: 465-472, 1952.
8. Hamerman, D., Hatch, F. T., Reife, A., and Bartz, K. W.: Nondialyzable Material in Normal Human Urine, J. Lab. & Clin. Med. **46**: 848-856, 1955.

STUDIES OF EXPERIMENTAL SULFHEMOGLOBINEMIA

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SULFHEMOGLOBIN, formed in circulating erythrocytes under the influence of drugs such as trinitrotoluene, is known to persist in vivo for long periods and in vitro as long as the cell remains intact.[1] Jope[2] therefore decided that sulfhemoglobin might be advantageously used to label red cells for survival studies, especially since it is formed rapidly and its formation ceases promptly when the causative agent is removed, so that the population of cells labeled is sharply defined. Furthermore neither the pigment itself nor the sulfur contained in sulfhemoglobin seem to be reutilized. Finally, according to Jope, sulfhemoglobinemia does not result in premature ("random") destruction of the labeled cells, nor is there any loss of sulfhemoglobin apart from cell destruction. These conclusions were drawn from his observations of 7 trinitrotoluene workers in whom he followed the disappearance of the pigment from the blood after withdrawal from contact. On plotting concentrations of sulfhemoglobin, expressed as per cent of that present on the day of withdrawal from contact against time, he found that all points from all seven subjects fell close to the same straight line whose slope indicated a lifespan of 116 ± 5 days. This way of plotting assumes that differences in average lifespan of red cells from different subjects are negligibly small, for Jope's standard deviation of 5 days covers this as well as experimental errors. The standard deviation for the latter alone may exceed five days even with such relatively accurate methods as tagging with Cr^{51},[3] and the rather scanty evidence available suggests that individual differences in lifespan may be fairly large. Analysis of the data given in figure 2 of Jope's paper shows that only four subjects were represented by more than two or three points each. For three of these subjects the points are reasonably linear and on extrapolation give lifespans between 110 and 120 days, but in the fourth case (represented by + in Jope's figure) the points fall on a smooth curve which is still well above the base line at 120 days and apparently indicates a lifespan longer than 160 days. The unusual longevity and the curvilinear disappearance raise the question of the accuracy of the method used for sulfhemoglobin determination and of the validity of the conclusions that random destruction is absent and cell survival normal. This condition would therefore differ from other forms of hemolytic anemia. A shortened survival time is indicated by the work of McKerns and Denstedt,[4] who produced sulfhemoglobinemia in rabbits by

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This investigation was supported in part by a research grant, H-1226, from the National Institutes of Health, Public Health Service.

Received for publication Jan. 6, 1956.

feeding acetophenetidin and sulfur; in these animals the pigment persisted about 10 days if the blood were transfused and about 20 days if it were not. This is much less than estimates of lifespan obtained in normal rabbits with isotopes: 50 and 67 days with radioiron[5, 6] and 61 days with N^{15}.[7] McKerns and Denstedt gave no details as to the shape of their disappearance curve. On the other hand, Dod, Bierman, and Shemkin[8] found that the sulfhemoglobin concentrations in the blood of rabbits and mice in which sulfhemoglobinemia had been induced by para-aminopropiophenone followed a logarithmic course for 43 days in rabbits and 19 days in mice. This does not correspond with the disappearance of normal cells which is linear but is compatible with the combination of linear and exponential disappearance seen in the presence of marked random destruction. The estimates of lifespan do not agree with those given above for the rabbit or with that of 25 days for the mouse.[5]

These discrepancies are probably to be attributed in large part to the methods used for sulfhemoglobin determination. Since this pigment has never been prepared in pure form, its optical constants must be determined in the presence of other hemoglobin derivatives and are consequently subject to greater error. The earlier estimates of Drabkin and Austin[9] are invalidated by the subsequent discovery that choleglobin and possibly also verdoglobin must also have been present in their solutions. Lemberg, Holden, Legge, and Lockwood[10] proposed a solution, but their constants are somewhat uncertain because of their use of a Hilger visual absorption spectrophotometer and also because of the possible presence of slight amounts of turbidity which, although invisible to the naked eye, affect spectrophotometric readings. Moreover they used only carbon monoxide derivatives for their measurements. Jope, because he wished to avoid carbon monoxide, compared the spectral absorption of bloods containing sulfhemoglobin before and after their conversion to the carboxy derivatives and derived equations for calculating sulfhemoglobin concentrations, making no correction for the possible presence of choleglobin since Lemberg and associates had shown that it was present only in negligible amounts in clinical sulfhemoglobinemia. However, they do not state how long an interval elapsed between cessation of the medication causing the sulfhemoglobinemia and the taking of the sample. This is of some importance, for choleglobin, like methemoglobin, disappears fairly rapidly from circulating blood, since it is easily converted into bile pigments.[10] Moreover the coefficient 0.00029 in Jope's equation[2] is obviously only about one-tenth of the correct value.* McKerns and Denstedt do not state how they estimated sulfhemoglobin, but Dod, Bierman, and Shemkin made a rough estimate using the ratio of the optical density of the blood diluted 1:10 at 620 mμ to that of the same blood diluted 1:100 at 540 mμ in the case of rabbits: for mouse blood 1:100 dilutions were used at both wave lengths, a base line being determined in untreated animals.

Because of these uncertainties and contradictions, it was decided to study the disappearance of experimentally produced sulfhemoglobinemia in animals, adapting the methods of Lemberg and associates to the Beckman photoelectric

*Jope in a personal communication to the authors agrees that this is incorrect.

spectrophotometer and following the destruction of red cells by two other, independent, methods: tagging with radioiron and measuring the excretion of bile pigments.

MATERIALS AND METHODS

Mongrel dogs weighing 8 to 17 kilograms were maintained on a diet of Purina Dog Chow, supplemented with cooked horse meat and bone scraps. They were kept free from worms and infections. New Zealand white rabbits weighing 3 to 4 kilograms were used, being fed Purina Rabbit Chow supplemented with oats and greens.

Sulfhemoglobin was produced by a variety of methods. Rabbits were given 750 mg. each of acetophenetidin and precipitated sulfur, daily, mixed with the food. Capsules containing 750 mg. of acetophenetidin or 600 mg. of sulfur each were mixed with the dogs' food, the usual daily dose being 3 capsules of each drug. Dosage was continued for one to four weeks. Some dogs were given, in addition to sulfur, acetanilid and some TNT (600 mg. capsules), while others received a single dose of 2 mg. of para-aminopropiophenone and 70 mg. of potassium sulfide per kilogram intravenously.

In certain animals, red cells were labeled with Fe^{55} (55 mc. per dog, 18 mc. per rabbit) administration of the iron being spread over three months or more, so that the whole red cell population was labeled. Sulfhemoglobinemia was then produced by administration of acetophenetidin and sulfur, and finally blood was transfused to other compatible dogs after blood grouping and crossmatching, according to the methods of Young and associates.[11] Reutilization of radioiron by the recipient animal was suppressed as far as possible by large doses of nonradioactive iron in the form of saccharated iron oxide, and the disappearance of both pigment and radioactivity was followed. Methods of tagging with iron and measuring radioactivity are described in detail elsewhere.[6]

Blood samples for sulfhemoglobin estimation were collected with 2.0 mg. per milliliter of potassium oxalate as anticoagulant; after thorough mixing, 0.2 ml. aliquots were transferred to 10 ml. volumetric flasks containing 3 to 4 ml. distilled water. To each of these was added in succession a trace of saponin, 4 ml. of M/15 phosphate buffer, pH 6.1, and distilled water to volume. To remove turbidity, the solutions were then centrifuged at 18,000 r.p.m. for 20 to 25 minutes at a temperature of 5° C. The solution was then divided into two measured portions, and each was treated for 3 to 4 minutes with carbon monoxide. A few milligrams of powdered sodium hyposulfite was added to one tube to reduce the pigments and to eliminate any methemoglobin that may have been formed. The optical densities were read immediately at 617 and 570 mμ. To the other tube, exactly 0.5 ml. of 20 per cent sodium hydroxide and a trace of sodium hyposulfite were added, and treatment with carbon monoxide was continued for two to three minutes more; optical densities were then measured at 630 and 570 mμ. By using blood whose hemoglobin concentration had been determined by oxygen capacity or iron analysis, we were able to calculate the specific extinction coefficients at these wave lengths for carboxyhemoglobin and CO-protohemochromogen. The values found (Table I) and the constants given by Lemberg and associates[10] for reduced CO-sulfhemoglobin and CO-cholehemochromogen were used to construct equations for calculating the amounts of the different pigments. When all three pigments were present, the concentration of cholegloblin (as CO-cholehemochromogen) in dog blood was calculated by the equation

$$\text{CHgb.} = 0.118\ E_{630} - 0.003\ E_{570} \quad (1)$$

and in rabbit blood by

$$\text{CHgb.} = 0.118\ E_{630} - 0.004\ E_{570} \quad (2)$$

The sum of the concentrations of reduced hemoglobin and sulfhemoglobin (as CO-protohemochromogen) in the same solutions is given, in dog blood, by

$$(\text{Hgb.} + \text{SHgb.}) = 0.116\ E_{570} - 0.053\ E_{630} \quad (3)$$

and, in rabbit blood, by

$$(\text{Hgb.} + \text{SHgb.}) = 0.138\ E_{570} - 0.063\ E_{630} \quad (4)$$

The concentrations of reduced hemoglobin and the sum of reduced sulfhemoglobin and cholegloblin were then determined on solutions saturated with carbon monoxide, using, for dog blood, the equations

$$\text{Hgb.} = 0.114\ E_{570} - 0.058\ E_{617} \quad (5)$$

$$(\text{SHgb.} + \text{CHgb.}) = 0.108\ E_{617} - 0.0036\ E_{570} \quad (6)$$

and for rabbit blood,

$$\text{Hgb.} = 0.118\ E_{570} - 0.062\ E_{617} \quad (7)$$

$$(\text{SHgb.} + \text{CHgb.}) = 0.112\ E_{617} - 0.0037\ E_{570} \quad (8)$$

TABLE I. EXTINCTION COEFFICIENTS FOR 1 PER CENT SOLUTIONS OF REDUCED CARBOXYHEMOGLOBIN AND CARBOXYPROTOHEMOCHROMOGENS WITH THE STANDARD ERROR OF THE MEAN

	DOG	RABBIT
Reduced COHgb		
No. of determinations	6	6
E_{570}	8.91 ± 0.04	8.70 ± 0.07
E_{617}	0.303 ± 0.011	0.305 ± 0.020
CO Protohemochromogen		
No. of determinations	6	6
E_{570}	8.72 ± 0.03	7.78 ± 0.34
E_{630}	0.226 ± 0.008	0.341 ± 0.095

Red cell destruction was also followed by studying pigment metabolism. Four dogs were kept in metabolism cages, and all fecal matter was collected over 4-day periods. A cleansing enema was given at the beginning and end of each collection period. Feces and enema fluid obtained during each period were then mixed thoroughly by a high-speed mechanical emulsifier and the fecal urobilinogen determined by the method of Schwartz, Shorov, and Watson.[12] Serum bilirubin was determined by the method of Ducci and Watson.[13] Hemoglobin was measured by oxygen capacity (Van Slyke) or by iron analysis using the method of Barkan and Walker.[15] Blood volume was estimated by the Evans Blue dye-hematocrit technique of Gibson and Evans.[14] Osmotic fragility studies were carried out by the method of Parpart and associates.[19]

RESULTS AND DISCUSSION

Values obtained by these methods for the specific extinction coefficients of carboxyhemoglobin and CO-protohemochromogen in the dog and rabbit are given in Table I. For the former Lemberg and Legge[16] gives $E_{568\text{-}572}$ as 13.7 to 15.0 which would be 9.4 to 10.2 for a 1 per cent solution, i.e., somewhat higher than our value. This might be due to a difference in species or in methods: high-speed centrifuging, for instance, might have removed minute amounts of substances causing turbidity. On the other hand, the difference between our values for dog and rabbit pigments at 570 mμ is statistically significant. In the case of CO-protohemochromogen the species difference at 570 mμ was also highly significant. Here Lemberg's value for human blood 8.6 lies between our values. No statistical differences were found for the coefficients at other wave lengths. While satisfactory amounts of sulfhemoglobin, up to about one-fifth of the total pigment, were produced in experimental animals, estimation of its concentration was not altogether satisfactory due to the presence of irregular errors the cause of which could not be traced, although slight degrees of turbidity were most probably involved.

The typical course of events in a dog following the administration of acetophenetidin and sulfur is shown in Fig. 1. Formation of sulfhemoglobin was accompanied by a marked decrease in the total circulating hemoglobin and the red cell volume. This was partly compensated for by a contraction of plasma volume, so that the decrease in number of red cells per cubic milli-

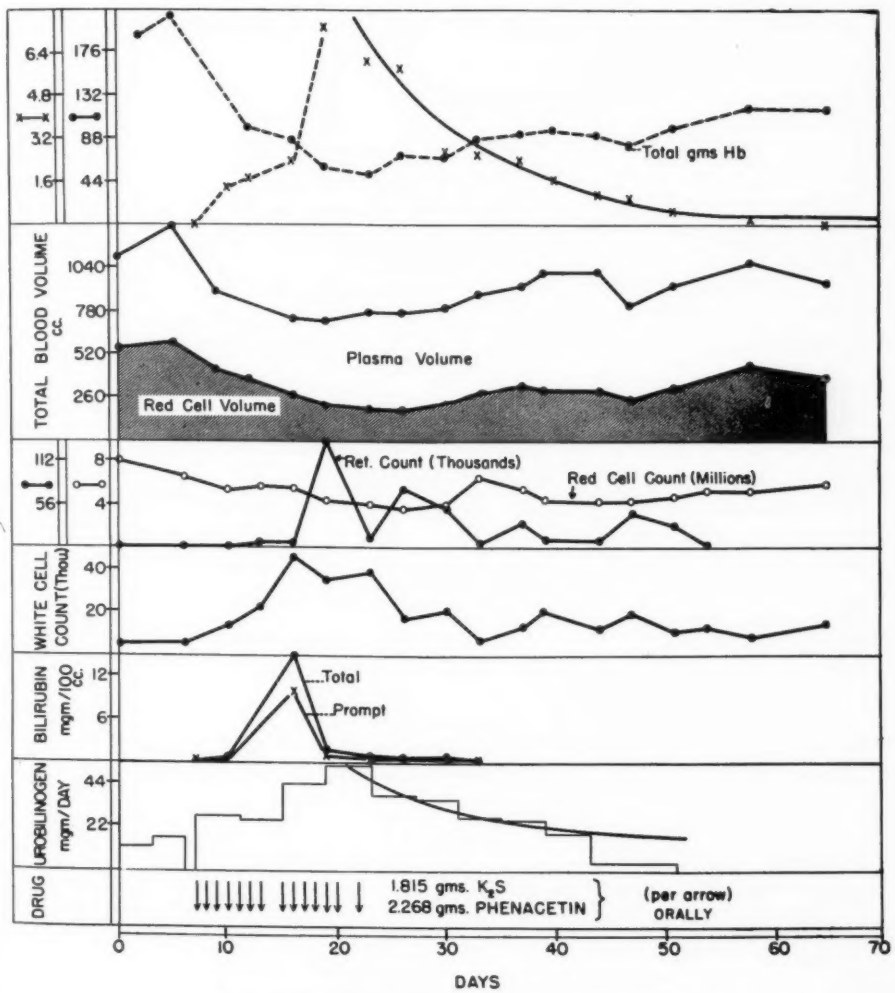

Fig. 1.—The production of sulfhemoglobinemia in a dog under the influence of acetophenetidin and sulfur and its disappearance on withdrawal of the drugs. The solid line in the highest section showing disappearance of sulfhemoglobin is drawn from equation 9 on the assumption of a potential lifespan of 107 days and a rate of random loss of 8 per cent a day. The solid line showing the decrease in urobilinogen excretion is drawn from equation 9 after differentiating and substituting the same values for T and k.

meter was less pronounced. At the same time there was an increase in urobilinogen excretion and in the bilirubin content of the blood. Beginning almost simultaneously with the fall in total hemoglobin, the reticulocyte and leukocyte counts rose. All this points to increased red cell destruction with an accompanying compensatory increase in hematopoiesis.

When the sulfhemoglobin content of the blood reached a level that was considered satisfactory, administration of drugs was stopped. Following

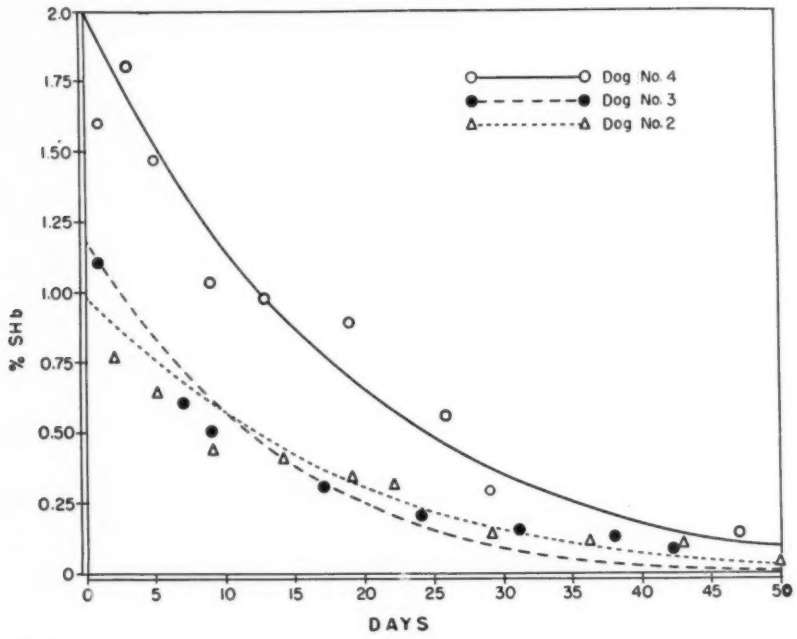

Fig. 2.—Three representative curves showing decay of sulfhemoglobin concentration in dog blood. The smooth curves are drawn from equation 9 using 107 days for T, and the following values for k: 0.046 (Dog 2), 0.066 (Dog 3), and 0.047 (Dog 4).

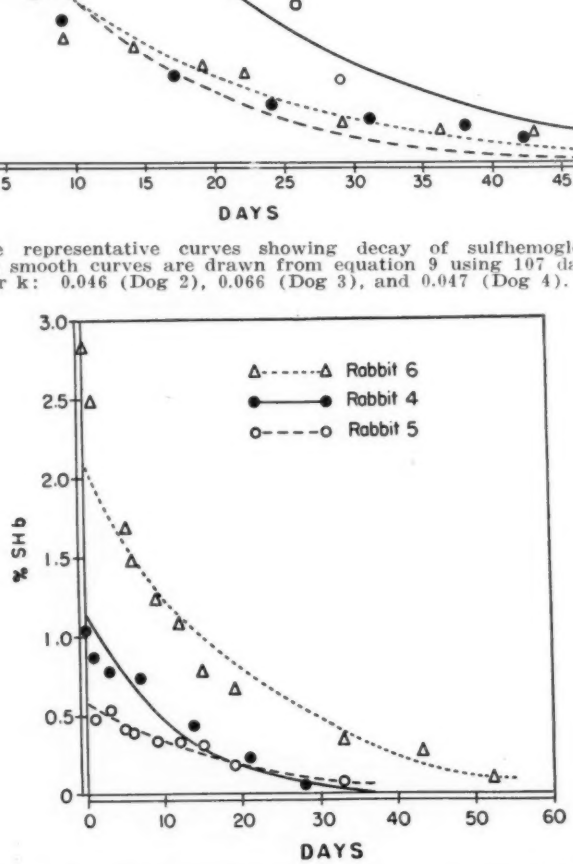

Fig. 3.—Three representative curves showing decay of sulfhemoglobin concentration in rabbit blood. The smooth curves are drawn from equation 9 using 67.6 days for T and the following values for k: 0.075 (Rabbit 4), 0.038 (Rabbit 5), and 0.049 (Rabbit 6).

this sulfhemoglobin disappeared gradually, in every case in curvilinear fashion. Examples of time-concentration curves for sulfhemoglobin may be seen

in Fig. 2; Fig. 3 illustrates this for rabbits. No instance of linear disappearance was seen. This suggests a combination of cell destruction from senescence, with random loss unrelated to the age of the cell. The latter might be a hemolytic process or merely a loss of the abnormal pigment by some first-order reaction. Consequently, disappearance might be expected to follow the equation[17]

$$S = S_0 \left(1 - \frac{t}{T}\right) e^{-kt} \qquad (9)$$

where S is the total quantity of sulfhemoglobin present on day t, S_0 that on day zero, T the potential lifespan, and k the constant of random loss. The smooth curves in Figs. 1, 2, and 3 are drawn from this equation, and it is clear that it affords a reasonably accurate description of the time curve of sulfhemoglobin disappearance. Departures from it appear to be random as might result from errors in sampling and in sulfhemoglobin estimation. This was confirmed by plotting the data on semilog paper. Data which fit equation 9 precisely when plotted in this way fall on a curve which, depending on the relative values of T and k, may either be indistinguishable from a straight line or convex from above downward to a greater or less degree. This is also true, within the limits of experimental error, for the data from these experiments and offers additional support for the hypothesis underlying equation 9.

Attempts were made to analyze the data in the same way as data previously obtained in this laboratory by tagging normal cells with Cr^{51},[3] but these were unsuccessful because the magnitude of the standard errors was such as to deprive the answers of all meaning. However, if an assumption is made as to the value of T, data can be fitted to equation 9 so as to obtain a value for k. This is not altogether independent of the value assumed for T, and some caution in interpretation is therefore necessary. Values for T determined in this laboratory[6] using Fe^{55} were used; 107 days for the dog and 67 days for the rabbit. Values calculated for k are given in Tables II *A* and II *B*, and the smooth curves in Figs. 1, 2, and 3 were drawn from these constants.

TABLE IIA. DOGS

DOG NO.	DRUG IN ADDITION TO SULFUR	TIME OF ADMINISTRATION DAYS	INITIAL SHb LEVEL (GM. %)	K (CONSTANT OF RANDOM DESTRUCTION)
1	Acetophenetidin	56	1.28	0.052
2	Acetophenetidin	58	1.15	0.046
3	Acetophenetidin	46	0.85	0.066
4	Acetophenetidin	54	1.55	0.047
5*	Acetophenetidin	Trans.	0.33	0.027
6	Acetophenetidin	37	1.56	0.060
7	Acetophenetidin	31	2.28	0.116
8	Acetophenetidin	43	0.79	0.080
9	Acetophenetidin	31	0.75	0.089
10*	Acetophenetidin	Trans.	0.39	0.041
11*	Acetophenetidin	Trans.	0.29	0.125
12	TNT	11	2.24	0.043
13	TNT	8	1.15	0.048
14	p-aminopropiophenone	1	1.19	0.047
15	p-aminopropiophenone	1	0.98	0.119

Assumed erythrocyte lifespan 107 days.
*After transfusion.

Curves fit the experimental points about as closely as could be expected in view of the magnitude of experimental error, and it is clear that the values chosen for the constants cannot be very far from the true values.

As the sulfhemoglobin levels of the blood decreased, there was a gradual fall in plasma bilirubin and in the rate of urobilinogen excretion. If the disappearance of sulfhemoglobin is due to the destruction of cells containing it rather than alteration of pigment within the cells, the rate of bile pigment excretion should be proportional to the rate of loss of sulfhemoglobin from the blood. The latter is the tangent to the time concentration curve and can be derived from equation 9 by differentiation. Because of the errors involved in collection of feces and in estimation of the pigment, no very close correspondence can be expected between an excretion curve derived from the blood data and the results of fecal analysis; nevertheless, it is clear that high rates of sulfhemoglobin loss are associated with high rates of excretion and that there is little or no excess output when the rates of loss are low. Plasma bilirubin figures give the same general picture.

TABLE IIB. RABBITS

RABBIT NO.	INITIAL SHGB LEVEL (GM. %)	K
1	0.41	0.039
2	1.03	0.058
3	0.67	0.048
4	1.04	0.075
5	0.72	0.038
6	2.85	0.049
7	2.27	0.036
8	2.27	0.033
9	1.29	0.036
10	0.49	0.033

Assumed erythrocyte lifespan 67.6 days.
All animals received acetophenetidin and sulfur.

Further evidence relating sulfhemoglobin disappearance to red cell destruction was obtained in experiments in which sulfhemoglobin was produced in cells previously labeled with Fe^{55}. Since there is no loss or exchange of iron with the intact red cell, the radioactive count, corrected for decay, could be used as a measure of the number of surviving labeled cells if it were not for the fact that radioiron, released by the destruction of the erythrocyte, is reutilized in the formation of fresh cells. For the same reason disappearance of radioiron will not follow equation 9. Fortunately the degree of reutilization can be kept small and relatively constant by the intravenous administration of nonradioactive iron.[5, 6] A theoretical expression for the time-course of disappearance of radioiron under these conditions can be derived in a way similar to that used to derive equation 9.[17] The radioactivity, N, present on any day t expressed as counts per minute corrected for decay is given by

$$N = N_0 - x - y + z \qquad (10)$$

where N_0 is the count on day zero, x the loss attributable to random destruction, y the loss from senescence, and z the gain due to reutilization. For experiments lasting somewhat less than the average potential lifespan T, y is

unaffected by reutilization, although decreased by random destruction. The reasoning previously used is therefore valid here and leads to the expression

$$y = \frac{N_0}{kT}(1 - e^{-kt}) \tag{11}$$

where k is the constant of random destruction. Since reutilization is proportional to the total rate of cell destruction

$$z = b(x + y) \tag{12}$$

where b is the reutilization constant. Therefore

$$N = N_0 - (1 - b)x - (1 - b)y \tag{13}$$

Random destruction in these experiments is almost entirely the result of the treatment used to produce sulfhemoglobinemia; there is little or none in normal dogs. Accordingly, the cells containing reutilized iron will not be affected and

$$dx/dt = k\ (N_0 - x - y) \tag{14}$$

The solution of these equations yields

$$N/N_0 = (1 - b)(1 - \frac{t}{T})e^{-kt} + b \tag{15}$$

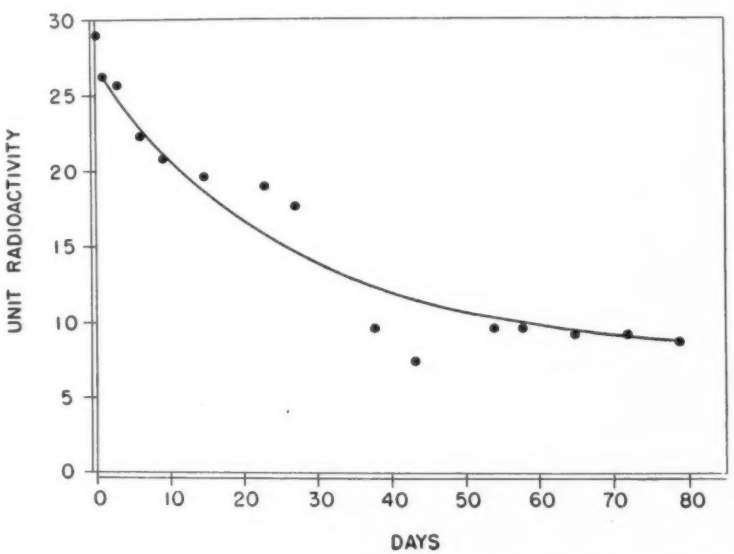

Fig. 4.—The rate of decay or radioactivity of sulfhemoglobin-containing cells tagged with Fe^{59}. Unit activity (C.P.M./ml. packed cells divided by C.P.M. of standard iron) is plotted against time in days. The smooth curve was drawn from equation 15 using the values: T = 107, k = 0.027, and b = 0.28.

This equation was applied to data from Dog 10. From the plot it was evident that b was approximately 0.28. An average lifespan T of 107 days was also assumed, and k was then calculated to be 0.027. The smooth curve in Fig. 4 is drawn from the equation using these values for the constants. It is clear that the curve fits the data about as well as could be expected and that therefore the values for the constants cannot be very far from the true ones. Be-

cause of irregularities in the data no closer approximation was attempted. The simultaneously determined constant of random loss for sulfhemoglobin was 0.041. This, although apparently somewhat greater than that for iron, is not significantly so by the F test. In the other dog, No. 11 in Table IIA, the rate of random destruction was much greater for both substances. Because of the presence of relatively high rates of random destruction in normal rabbits,[6, 7] no analysis of their curves was attempted, but there was obviously a close correspondence between the rates of disappearance of both labels. It may therefore be concluded that the rate of random loss of sulfhemoglobin is closely related to and probably identical with that of random destruction of the cells as measured with radioiron and that the disappearance of sulfhemoglobin is due to destruction of the cells containing it.

The cause of random destruction lies in alterations produced in the cell rather than in the plasma. This is shown by transfusion experiments. Sulfhemoglobin-containing cells transfused into normal animals show about the same degree of random destruction as untransfused cells (Fig. 5 and Table IIA).

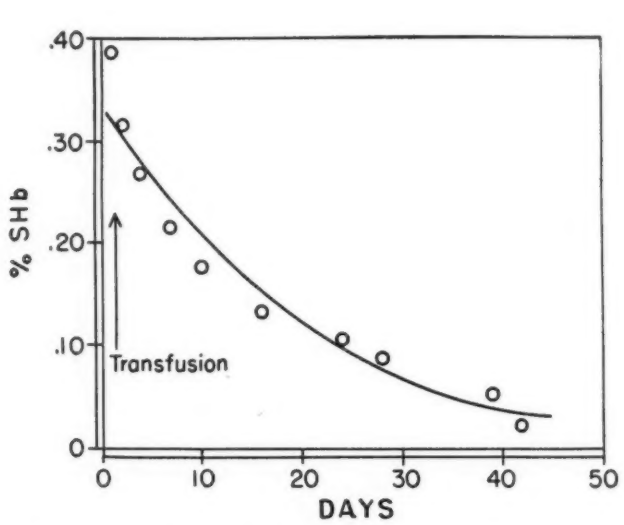

Fig. 5.—The rate of loss of sulfhemoglobin after transfusion of the cells containing it to a dog which had not been treated with hemolytic drugs. The smooth curve is drawn from equation 9 using T = 107 and k = 0.041.

The cause of random destruction does not lie in any decrease in the ability of the cell to withstand changes in osmotic pressure, for repeated determinations of osmotic fragility in the presence and absence of sulfhemoglobin in both dogs and rabbits show no difference (Fig. 6). This confirms Lemberg, Holden, Legge, and Lockwood,[10] who showed that although extensive hemolysis occurred on washing cells in which sulfhemoglobin had been produced by acetanilid and sulfur, the hemolysate contained the same percentage of sulfhemoglobin and choleglobin as the unwashed cells.

While the presence of sulfhemoglobin is visible evidence of damage done to the cell, it is not necessarily the only change that has occurred and the ques-

tion arises whether sulfhemoglobin itself is the cause of the instability which manifests itself as random red cell destruction. Comparison of the constants of random destruction with the corresponding initial sulfhemoglobin concentrations (Tables II*A* and II*B*) shows that they are apparently unrelated. This can only mean that sulfhemoglobin itself is probably not the cause of the destruction but that it, like methemoglobin, which is formed at the same time, is rather one of the signs of damage. Van Loon and Clark,[18] working with acetanilid and acetophenetidin, interpret their findings in much the same way and suggest that "the ability of the drug to produce methemoglobinemia and hemolytic anemia may be coincident actions" rather than related as cause and effect. In their experiments little or no sulfhemoglobin was found in the blood. We have confirmed this in experiments in which acetophenetidin or acetanilid was administered without sulfur to dogs in quantities sufficient to produce a fall in red cell count and hemoglobin level. In these animals the blood contained methemoglobin but not sulfhemoglobin. The latter cannot therefore be the cause of the random destruction. Methemoglobinemia, however, is very transient; like other authors[18] we have observed that it disappears within three or four days after the last dose of the drug producing it.

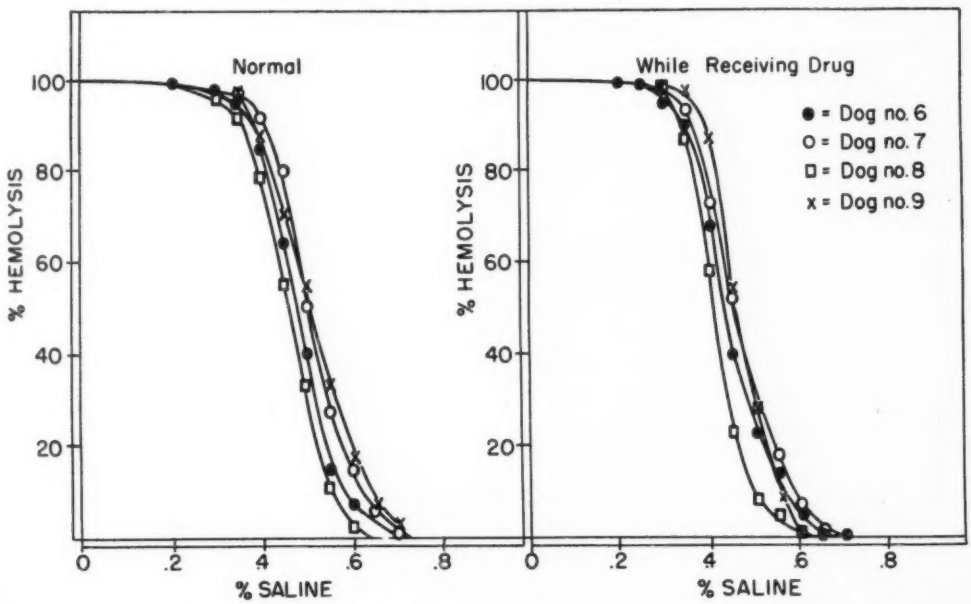

Fig. 6.—Osmotic fragility curves of dog blood before and after administration of sulfur and acetophenetidin. The presence of sulhemoglobinemia has no effect on osmotic fragility.

Random destruction, on the other hand, persists for many weeks, in fact until all the cells that were exposed to the drug have disappeared. In view of this it is difficult to see how methemoglobin could cause the destruction of these cells. The rate at which sulfhemoglobinemia is produced varies with different drugs. It is relatively slow with acetophenetidin and very rapid with p-aminopropiophenone, TNT being intermediate. But the concomitant

random destruction apparently occurs at a rate independent of this, so that with acetophenetidin, which must be administered over a long period, there is much more hemolysis for the same level of sulfhemoglobinemia than with the other drugs. Fig. 7 illustrates an experiment in which sulfhemoglobin production was induced in the same animal by two different drugs at different times. In producing a smaller degree of sulfhemoglobinemia (0.75 Gm. per cent), acetophenetidin damaged cells to the extent that the rate of random destruction was 0.073. TNT on the other hand resulted in a sulfhemoglobin level of 2.2 Gm. per cent and a random destruction rate of 0.043.

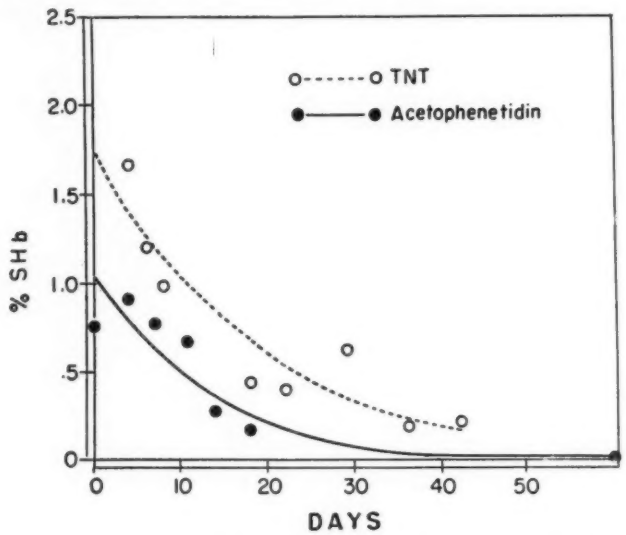

Fig. 7.—Disappearance of sulfhemoglobin from plasma of a dog in which it was induced successively by acetophenetidin (solid circles) and TNT (open circles) with sulfur. The smooth curves were drawn from equation 9 using T = 107 and k = 0.073 (acetophenetidin: solid line) and k = 0.043 (TNT: broken line).

SUMMARY

Sulfhemoglobinemia was produced in dogs and rabbits by administration of sulfur together with acetophenetidin, TNT, or p-aminopropiophenone. The sulfhemoglobin content of the blood, the unaltered hemoglobin, red cell, reticulocyte and white cell counts, serum bilirubin content, and bile pigment excretion were followed during the formation of the sulfhemoglobin and until its disappearance after the drugs were discontinued or after transfusion. In addition, cells containing sulfhemoglobin were followed by tagging with radioiron. Formation of sulfhemoglobin occurred most rapidly on administration of p-aminopropiophenone, least rapidly with acetophenetidin. All these drugs produced hemolysis at a rate apparently unrelated to sulfhemoglobin formation. Disappearance of sulfhemoglobin was due to a random destruction process as well as senescence of the cells containing it. There is no evidence for accelerated senescence. Quantitative estimation of sulfhemoglobin in blood is discussed, and new values are given for the specific extinction coefficients of reduced carboxyhemoglobin and CO-protohemochromogen.

REFERENCES

1. Drabkin, D. L.: Metabolism of the Hemin Chromoproteins, Physiol. Rev. **31:** 345-431, 1951.
2. Jope, E. M.: The Disappearance of Sulphhaemoglobin From the Blood of TNT Workers in Relation to the Dynamics of Red Cell Destruction, Brit. J. Indust. M. **3:** 136-142, 1946.
3. Eadie, G. S., Brown, I. W., Jr., and Curtis, W. G.: The Potential Life Span and Ultimate Survival of Fresh Red Blood Cells in Normal Healthy Recipients as Studied by Simultaneous Cr^{51} Tagging and Differential Hemolysis, J. Clin. Invest. **34:** 629-636, 1955.
4. McKerns, K. W., and Denstedt, O. L.: Studies With Sulfhemoglobin-labelled Erythrocytes. The Use of Sulfhemoglobin for Labelling Erythrocytes. The Preservation of the Formed Elements and of the Proteins of the Blood. Boston, Mass. 1949, American National Red Cross.
5. Harrison, A., Burwell, E. L., and Finch, C. A.: Erythrocyte Life Span in Small Animals, Fed. Proc. **10:** 357, 1951.
6. Brown, I. W., Jr., and Eadie, G. S.: An Analytical Study of In Vivo Survival of Limited Populations of Animal Red Blood Cells Tagged With Radioiron, J. Gen. Physiol. **36:** 327-343, 1953.
7. Neuberger, A., and Niven, J. S. F.: Haemoglobin Formation in Rabbits, J. Physiol. **112:** 292-310, 1951.
8. Dod, K. S., Bierman, H. R., and Shemkin, M. B.: The Disappearance of Sulfhemoglobin in the Blood as a Measure of the Life Span of Erythrocytes in Normal Rabbits and Mice and in Tumor-bearing Mice, J. Nat. Cancer Inst. **11:** 1093-1103, 1951.
9. Drabkin, D. L., and Austin, J. H.: Spectrophotometric Studies. II. Preparations From Washed Blood Cells; Nitric Oxide Hemoglobin and Sulfhemoglobin, J. Biol. Chem. **112:** 51-65, 1935.
10. Lemberg, R., Holden, H. G., Legge, J. W., and Lockwood, W. H.: Studies on Sulphhaemoglobin, Australian J. Exper. Biol. & M. Sc. **20:** 161-167, 1942.
11. Young, L. E., Erwin, D. M., and Yuile, C. L.: Hemolytic Reactions Produced in Dogs by Transfusion of Incompatible Dog Blood and Plasma, Blood **4:** 1218-1231, 1949.
12. Schwartz, S., Shorov, V., and Watson, C. J.: Studies of Urobilinogen. IV. The Quantitative Determination of Urobilinogen by Means of the Evelyn Photoelectric Colorimeter, Am. J. Clin. Path. **14:** 598-604, 1944.
13. Ducci, H., and Watson, C. J.: The Quantitative Determination of the Serum Bilirubin With Speial Reference to the Prompt-reacting and the Chloroform Soluble Types, J. LAB. & CLIN. MED. **30:** 293-299, 1945.
14. Gibson, J. G., II, and Evans, W. A., Jr.: Clinical Studies of the Blood Volume. I. Clinical Application of a Method Employing the Azo Dye "Evans Blue" and the Spectrophotometer, J. Clin. Invest. **16:** 301-316, 1937.
15. Barkan, G., and Walker, B. S.: Determination of Serum Iron and Pseudohemoglobin Iron With O-Phenanthraline, J. Biol. Chem. **135:** 37-42, 1940.
16. Lemberg, R., and Legge, J. W.: Hematin Compounds and Bile Pigments, New York, 1949, Interscience Publishers.
17. Eadie, G. S., and Brown, I. W., Jr.: Red Blood Cell Survival Studies, Blood **8:** 1110-1136, 1953.
18. Van Loon, E. G., and Clark, B. B.: Observations on the Hematologic Actions of Acetanilid and Acetophenetidin in the Dog, J. LAB. & CLIN. MED. **29:** 942-956, 1944.
19. Parpart, A. K., Lorenz, P. B., Parpart, E. R., Gregg, J. R., and Chase, E. M.: The Osmotic Resistance (Fragility) of Human Red Cells, J. Clin. Invest. **26:** 636, 1947.

AN EVALUATION OF THE FECAL RECOVERY METHOD FOR DETERMINING INTESTINAL ABSORPTION OF COBALT^{60}-LABELED VITAMIN B_{12}

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THREE methods are available to measure intestinal absorption of vitamin B_{12}, with use of radioactive (cobalt^{60}-labeled) vitamin B_{12}. The first, reported by Heinle and associates,[1] in 1952, involves collection of stools after oral administration of a test dose. The difference between radioactivity recovered in the feces and the amount administered represents the amount absorbed. In the second method, devised by Schilling[2] in 1953, a test dose is given followed by parenteral injection of 1,000 mcg. of nonradioactive B_{12}. This results in "flushing out" of some of the absorbed radioactivity into the urine where it can be counted. A third method, reported by Glass and coworkers,[3] consists of surface scintillation counting over the liver several days after administration of a test dose of radioactive B_{12}. If absorption has occurred, radioactivity will be detected over the liver where most of the B_{12} is concentrated.

The urinary excretion test is simpler to carry out than the fecal recovery test. Furthermore, it does not involve work with stools, which may be objectionable to laboratory and ward personnel. Therefore it has been used more extensively.[4-6] However, in respect to quantitative determination of absorption it would appear that the most direct means would be to subtract radioactivity eliminated in the feces from the amount administered by mouth.

Four hundred and eighty-six fecal excretion tests have been performed in this laboratory. The first 146 tests were carried out by transferring each stool to a porcelain crucible and then drying it, ashing in a muffle furnace, and counting the ashed residue made up to 25 ml. Because of the cumbersome features of this method, a simpler technique was devised which does not involve drying and ashing or transfer of feces from one container to another. Three hundred and forty tests have been carried out by this latter direct-counting method. Results of the test in pernicious anemia and total gastrectomy and the effect of intrinsic factor in these two conditions, as well as in normal individuals, have been previously reported.[7-9] The effect of antibiotics on absorption in a variety of conditions has also been reported.[10, 11]

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Supported in part by grants from Merck & Co., Rahway, N. J., Organon Inc., Orange, N. J., and The Upjohn Co., Kalamazoo, Mich.

Received for publication Jan. 7, 1956.

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This report has three objectives: (1) to describe the direct counting method; (2) to report the results of two or more tests carried out in the same patient under similar conditions, in order to define the limits of reproducibility of the method; and (3) to define its usefulness as a diagnostic test of intrinsic factor deficiency.

DESCRIPTION OF THE METHOD

The tests were carried out as follows: The test dose of Co^{60}-B_{12},* usually 0.5 mcg., was administered orally in water, between meals. Each dose contained approximately 0.1 μc of radioactivity. Nonradioactive, crystalline vitamin B_{12} was added in varying amounts whenever the absorption of larger doses was tested. For example, if 2.0 mcg. was to be used as the test dose, 1.5 mcg. of nonradioactive, crystalline B_{12} was added to 0.5 mcg. of Co^{60}-B_{12}. All stools were passed by the patient directly into quart ice cream containers of constant dimension. The contents of each container was made up to 400 ml. with water and mixed with an electrically driven stirring rod for 15 minutes. The container was then placed directly over the crystal and counted to an appropriate number of counts, usually 3,200. A standard solution of radioactive B_{12} was made up in 400 ml. of water in an identical carton and counted every day. Elimination was considered complete when the last stool in which a count of less than 1½ times background was obtained. The amount of radioactivity in each stool was calculated from the standard, and the amounts in each day's collection were cumulated to determine the total recovery of radioactivity.

The usual number of stools required to complete the test was 3 or 4 (120 tests each). The least number was 1 (2 tests), and the greatest number was 9 (4 tests). At least one stool was collected after the last detectable radioactivity to ensure complete elimination. In constipated patients two stools were usually obtained after radioactivity was no longer present. In some instances a cathartic was administered.

The crystal and shield used are shown in Fig. 1.† The lead shield was 2 inches thick. A flat, sodium iodide, thallium-activated crystal, 1¾ × 2 inches, was employed. A plastic bag was placed inside the shield covering the crystal to obviate the possibility of radioactive fecal material contaminating the apparatus.

The chief source of error inherent in the method is incomplete stool collection. This can be minimized by selection of reliable subjects, explanation of the collection procedure in detail to the subject, collection of specimens in carefully labeled and dated cartons on a well-run ward where the nurses and aides are familiar with the problem, and by checks daily with the patient to see that he is cooperating fully. For these reasons the studies were mainly carried out on the research ward.

The stools, placed under a hood, were well homogenized with an electric-driven stirrer for 15 minutes so that the particles were evenly dispersed. Settling of heavier particles with a subsequent rise in count may occur, and expansion caused by gas formation with a consequent lowering of the count does occur if stools are left to stand. However, these errors will be avoided if each stool is counted within an hour after mixing.

In making up the specimen to a constant volume, water is added to the container in which it was collected to a previously measured 400 ml. mark. Although by this method the final volume may not be precisely 400 ml., the error introduced by a variation of as much as 25 ml. has been found to be less than 4 per cent of total excretion.

In order that it might be determined that mixing of the specimens by the method described resulted in even dispersion, six stools containing varying amounts of the administered test dose were each divided into four equal aliquots. The results (Table I) showed very even distribution of the radioactivity, the variation being less than 1 per cent in each instance.

*Kindly supplied by Dr. Charles A. Rosenblum, Merck and Co., Rahway, N. J.

†Constructed by Reed-Curtis Nuclear Industries, Inc., 307 Culver Boulevard, Playa del Rey, Calif.

TABLE I. COMPARISON OF COUNTS FROM ALIQUOTS OF HOMOGENIZED STOOLS

¼ ALIQUOTS PER CENT OF DOSE	GREATEST VARIATION PER CENT OF DOSE
12.5, 12.5, 13.1, 12.8	0.6
13.8, 13.7, 13.3, 13.2	0.6
5.7, 6.2, 6.3, 6.4	0.7
10.8, 10.3, 10.8, 10.8	0.5
10.9, 10.8, 11.0, 11.4	0.6
2.9, 2.9, 2.7, 2.8	0.2

Thirteen recovery experiments were carried out in which Co^{60}-B_{12} was mixed with nonradioactive feces. The specimens consisted of 8 stools of normal consistency, 2 liquid stools, and 3 stools containing large amounts of fat from total gastrectomy patients. Between 93 and 102 per cent of the Co^{60}-B_{12} was recovered in the thirteen experiments. Excluding the fatty stools, recovery was between 96 and 100 per cent.

To minimize the variables which might be introduced by B_{12}-containing foods and stimulation of gastric secretion, the test dose was administered either fasting or between meals.

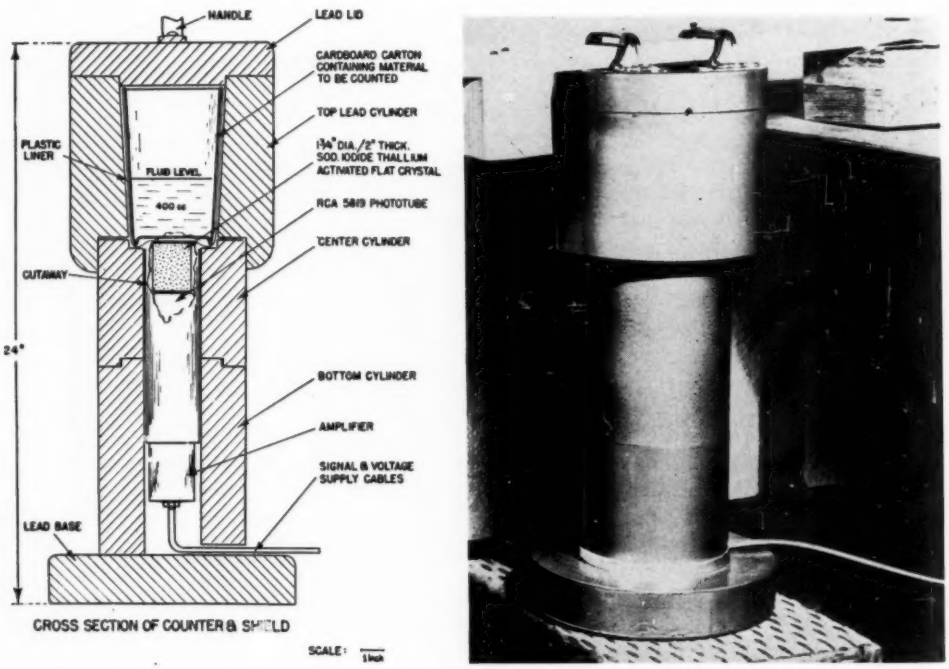

Fig. 1.—Cross-section diagram (left) and photograph (right) of scintillation counter and shield used in fecal recovery test for Co^{60}-labeled vitamin B_{12}.

In order to study the possibility that B_{12} might be excreted into the bowel, two patients with terminal carcinoma were given parenteral injections of Co^{60}-B_{12} and fecal radioactivity measured over several days. The first patient was given 9.4 mcg. containing 2.0 μc of radioactivity. One and seven-tenths per cent of the radioactivity was excreted in the feces over an 11-day period. The second patient was given 0.5 mcg. containing 0.106 μc, and 2.5 per cent of this amount was excreted in the feces. A third patient with a T-tube in the common bile duct was given 2.0 mcg. containing 0.1 μc by mouth. Forty-two per cent was recovered in the feces, but no radioactivity could be obtained in

the biliary secretions. These experiments indicate that injected and orally administered Co^{60}-B_{12} may be excreted into the bowel in small but insignificant amounts and that intestinal absorption is not followed by excretion into the bile.

Of the 340 tests carried out with use of the method described, 34, or 10 per cent, were discarded as unsatisfactory, due to inaccurate stool collections. Either the patient did not collect all stools or submitted only a portion of a specimen, or collection was stopped before all radioactivity had been recovered.

RESULTS

A. Patients in Whom Tests Were Repeated at Least Once.—

1. Instrinsic factor deficiency (Table II, A): In 14 patients with addisonian pernicious anemia or a total gastrectomy in whom 21 repeat tests under the same conditions were carried out, the maximum variation from test to test

TABLE II. VARIATION IN FECAL RECOVERY OF Co^{60}-B_{12} IN TESTS REPEATED IN SAME SUBJECT UNDER SIMILAR CONDITIONS

A. Intrinsic Factor Deficiency (Addisonian Pernicious Anemia and Total Gastrectomy)

PATIENT	AGE	DIAGNOSIS	DOSE OF Co^{60}-B_{12} (MCG.) AND CONDITIONS OF TEST	PER CENT RADIOACTIVITY RECOVERED IN FECES			
				TEST 1	TEST 2	TEST 3	VARIATION
B. A.	61	Total gastrectomy	5.0	97	92	83	14
M. E.	57	Total gastrectomy	0.5*	100	100	100	0
			0.5 with 50 ml. gastric juice*	8	20		12
E. C.	32	Partial gastrectomy, macrocytic anemia (B_{12} def.)	0.5	100	97		3
G. L.	62	Pernicious anemia	0.5*	100	100		0
M. M.	62	Total gastrectomy	5.0	82	100	91	18
D. M.	79	Pernicious anemia	0.5	90	93		3
D. O.	88	Pernicious anemia	0.5	86	85		1
F. P.	62	Total gastrectomy	0.5*	100	100		0
J. R.	76	Pernicious anemia	0.5*	100	97		3
			0.5	89	88		1
M. Sp.†	56	Total gastrectomy	0.5	81	87		6
G. S.	43	Total gastrectomy	0.5*	95	96		1
			5.0	81	88	91	10
			0.5 with intrinsic factor assay fraction	65	77		12
M. Sw.	69	Pernicious anemia	0.5*	99	99		0
			0.5 with Neomycin	100	100		0
			0.5 with Aureomycin	84	80		4
J. T.	74	Pernicious anemia	0.5 with intrinsic factor assay fraction	43	40		3
			0.5 with Aureomycin	88	96		8
J. C. T.	59	Total gastrectomy	0.5	100	96		4

Maximum variation = 18 per cent.
Average variation = 5 per cent.

*Test performed by the original drying-ashing method. All other tests were performed by the method of counting the entire stool made up to constant volume of 400 ml. as described.

†Three centimeters of cardia left at operation. A Schilling test, with use of 0.5 mcg. Co^{60}-B_{12} as the test dose, gave 3.5 per cent urinary excretion of radioactivity.

was 18 per cent, with an average of 5 per cent. The variation of 18 per cent was in a patient (M. M.) given 5.0 mcg. of Co^{60}-B_{12}. In the patients given 0.5 mcg. alone, the greatest variation was 6 per cent.

2. Patients with intestinal disease (Table II, B): The fecal recovery in this group of 9 patients with 15 repeat tests was less as a rule than in the patients with intrinsic factor deficiency, but it was greater than in the normals and patients with miscellaneous diseases. The maximum variation was 21 per cent with an average of 11 per cent. The greater variation from test to test in this group is presumably due to altered bowel motility. Most of the patients had diarrhea.

The effect of diarrhea and constipation on fecal elimination of Co^{60}-B_{12} is difficult to evaluate. In the patient (H. H.) with regional enteritis and macrocytic anemia responding to folic acid (Table II, *B*), who had moderate diarrhea, fecal elimination decreased markedly when the patient was given codeine. Yet in the patient (W. M.) with small bowel diverticulosis, a "blind loop," and macrocytic anemia, there was no decrease when the patient's diarrhea was controlled with codeine.

In constipated patients, fecal elimination of radioactivity is much slower than in patients with diarrhea. However, when the last stool shows no radio-

TABLE II—CONT'D

B. Intestinal Disease

PATIENT	AGE	DIAGNOSIS	DOSE Co^{60}-B_{12} (MCG.) AND CONDITIONS OF TEST	PER CENT RADIOACTIVITY RECOVERED IN FECES				
				TEST 1	TEST 2	TEST 3	TEST 4	VARIATION
J. B.	44	Regional enteritis, steatorrhea, macrocytic anemia (B_{12} def.)	0.5 with Aureomycin	85	94	89		9
C. B.	55	Pancreatic steatorrhea	0.5	76	71			5
			0.5 with Aureomycin	45	55	46		10
B. D.	42	Nontropical sprue, macrocytic anemia (B_{12} def.)	0.5 with Aureomycin	61	52			9
H. H.	30	Regional enteritis, macrocytic anemia (folic acid deficiency)	0.5	64	71			7
E. M.	31	Nontropical sprue (normocytic anemia)	0.5	44	23			21
			0.5 with Aureomycin	43	27			14
W. M.	59	Blind loop, macrocytic anemia (B_{12} def.)	0.5	98	84	91	99	15
			0.5 with Aureomycin	44	65			21
			0.5 with Achromycin	63	60	60		3
			0.5 with Neomycin	93	92	98		6
H. S.	31	Regional enteritis	0.5	77	81			4
J. S.	23	Blind loop, macrocytic anemia (B_{12} def.)	0.5	59	77	59	72	18
			0.5 with Aureomycin	25	15			10
P. T.	24	Regional enteritis	0.5	70	67	80		13

Maximum variation = 21 per cent.
Average variation = 11 per cent.

activity it can be assumed that excretion is complete, because in six such patients who were given a strong cathartic no additional radioactivity could be recovered in several subsequent stools.

3. Normals and patients with miscellaneous diseases (Table II, C): In this group of 19 individuals with 27 repeat tests the maximum variation was 31 per cent with an average of 8 per cent. However, in 11 of the 27 tests, doses of 2.0 or 5.0 mcg. were used. The maximum variation with test doses of 0.5 mcg. was 19 per cent, the average being 7 per cent. With doses greater than 0.5 mcg., fecal recovery is much higher with a wider range of reproducibility.

TABLE II—CONT'D

C. Normals and Miscellaneous Diseases

PATIENT	AGE	DIAGNOSIS	DOSE Co^{60}-B_{12} (MCG.) AND CONDITIONS OF TEST	PER CENT RADIOACTIVITY RECOVERED IN FECES			
				TEST 1	TEST 2	TEST 3	VARIATION
S. B.	33	Rheum. arthritis	0.5	25	26		1
E. C.	64	Cerebral accident (old)	0.5	27	25		2
E. C.	60	Diabetes	0.5	38	31		7
L. C.	62	Diabetes	0.5	37	56		19
A. G.	35	Chronic urticaria	2.0*	35	57		22
M. G.	30	Normal	0.5	20	28		8
			5.0*	74	87		13
J. H.	48	Normal	0.5*	32	29		3
			5.0*	64	71		7
D. H.	60	Duod. ulcer	5.0	59	50		9
T. K.	32	Coccidiomycosis (patient nontoxic)	0.5	32	30		2
			2.0	82	64	51	31
C. K.	48	Hypogonadism	0.5*	27	30		3
			5.0*	78	67		11
J. M.	35	Rheum. arthritis	0.5	36	47		11
			0.5 with Neomycin	74	75		1
M. M.	81	Gastritis	0.5	17	5		12
A. M.	39	Dermatitis	2.0*	54	59		5
M. P.	35	Rheum. arthritis	0.5	26	40	30	14
			2.0	74	75		1
			5.0	83	72		11
R. R.	64	Rheum. arthritis	2.0*	66	68	69	3
			5.0*	94	96		2
E. S.	62	Panhypopituitarism	0.5	48	54		6
W. W.	75	Achlorhydria, cerebral arteriosclerosis	0.5	63	68		5
H. W.	32	Diabetes	0.5	40	43		3
L. O.	59	Achlorhydria, anemia, (?) type	0.5	61	70		9

Maximum variation = 31 per cent.
Average variation = 8 per cent.
Excluding tests in which mort than 0.5 mcg. were given,
the maximum variation = 19 per cent
the average variation = 7 per cent.

The results of the repeated tests in the 28 patients with intestinal and miscellaneous diseases are similar to those of Callender and Evans.[6] These authors found a range of difference from 0 to 19 per cent in repeated tests performed in 38 patients.

B. Fecal Excretion in Patients With Intrinsic Factor Deficiency Compared With Controls.—If the fecal elimination test is to be reliable as a diagnostic tool for intrinsic factor deficiency, recovery of radioactivity in the test dose should always be significantly less in patients with a functioning gastric mucosa than in those without intrinsic factor activity. Table III gives the results of all of the tests which have been performed in individuals in whom there was no clinical evidence of gastrointestinal disease or vitamin B_{12} deficiency. They were either normal people or patients with a variety of hos-

TABLE III. FECAL RECOVERY OF Co^{60}-B_{12} IN CONTROL* INDIVIDUALS

TEST DOSE			
0.5 MCG.	2.0 MCG.	5.0 MCG.	10.0 MCG.
38 TESTS IN 30 PATIENTS	23 TESTS IN 21 PATIENTS	14 TESTS IN 13 PATIENTS	7 TESTS IN 6 PATIENTS
PER CENT RECOVERY			
57	96	98	100
56	92	83	93
54	89	81	88
47	82	78	85
45	76	75	82
45	75	74	70
43	74	72	66
41	69	72	
40	64	64	
40	56	60	
38	53	59	
38	52	59	
37	52	57	
36	51	50	
36	50		
34	50		
33	48		
32	35		
32	35		
31	30		
30	27		
30	22		
29	17		
28			
27			
27			
27			
26			
26			
25			
25			
25			
20			
19			
18			
17			
12			
5			
Average: 34	Average: 54	Average: 72	Average: 84

*Patients with a variety of diseases but without intestinal disease, anemia, fever, sepsis, or malnutrition.

pital diseases. None had anemia, acute infection, fever, intestinal disease, or malnutrition. With a test dose of 0.5 mcg. in 38 tests in 30 individuals, the maximum recovery was 57 per cent and the least was 5 per cent. The average was 34 per cent. The results in general are in close agreement with those reported by Callender and associates.[12]

With test doses of 2.0, 5.0, and 10.0 mcg. the recovery of radioactivity increases to average levels of 52, 72, and 84 per cent, respectively. This limitation to the absorption of vitamin B_{12} has been discussed by Callender,[14] Glass,[13] and ourselves.[9]

The results of the fecal elimination test, with use of a test dose of 0.5 mcg. in 13 patients with pernicious anemia and in 11 patients with total gastrectomy are shown in Table IV. In this group of 24 patients, 34 tests were performed. The lowest recovery was 78 per cent in pernicious anemia and 81 per cent in total gastrectomy, the average for the two groups being 94 per cent.

TABLE IV. FECAL RECOVERY OF Co^{60}-B_{12} IN PATIENTS WITH INTRINSIC FACTOR DEFICIENCY (TEST DOSE OF 0.5 MCG.)

PERNICIOUS ANEMIA	TOTAL GASTRECTOMY
19 TESTS IN 13 PATIENTS	15 TESTS IN 11 PATIENTS
PER CENT RECOVERY	
100	100
100	100
100	100
99	100
99	100
97	100
94	100
93	96
92	96
90	95
89	95
88	93
87	87
86	87
85	81
84	
84	
80	
78	
Average: 94	

DISCUSSION

The fecal elimination test appears to be the most direct method for determining quantitative absorption of vitamin B_{12}. As indicated in Table II, *A*, *B*, and *C*, the results can be reproduced with a fair degree of correlation, provided a test dose of 0.5 mcg. of Co^{60}-B_{12} is employed. Thus it is a reliable test for the study of factors involved in the mechanisms of absorption where quantitative changes are to be determined. As a research tool in this regard, it is to be preferred to other methods where absorption is measured either over the liver or in the urine, after the vitamin has crossed the intestinal membrane and passed through the body tissues.*

*Comments relative to comparison of the urinary and fecal tests are made in the light of experience with 19 urinary excretion tests carried out in this laboratory, and 103 tests performed in the Research Laboratory of the Syracuse V.A. Hospital by Dr. Halsted, in addition to published reports. We have had no experience with the hepatic-uptake method.

The technique described here for the fecal recovery method is simple and the result for a given stool specimen can be obtained within a half-hour after it is passed by the patient. Transfer of stool from one container to another is not necessary, provided the bulk of the specimen is less than 400 ml. The disadvantage of the test is obvious: namely, the necessity for complete cooperation of the patient and ward personnel to insure accurate collection of all stools over a period of several days. Thus it is desirable to limit the use of the method to a special research ward.

The apparatus and technique should be applicable to measurements of fecal radioactivity in absorption studies with other gamma emitting isotopes such as Fe^{59}.

As a clinical test for the diagnosis of intrinsic factor deficiency, measurement of the actual amount of B_{12} absorbed is less important than the determination of whether it can be absorbed at all. The urinary excretion test is preferable for the reason that a complete 24-hour urine collection is much more easily obtained than a complete fecal collection of several days. If a stool is lost, the result may be misleading. Furthermore the fecal recovery method takes a week for completion whereas the urinary excretion test can be completed in 24 hours. If no radioactivity is found in the urine, one can assume that none has been absorbed from the gut. As a diagnostic test for intrinsic factor deficiency in patients with macrocytic anemia, there is the disadvantage that one is forced to give a parenteral injection of a large amount of vitamin B_{12} before an etiologic diagnosis is established, which may be undesirable. Intrinsic factor fractions can be tested in patients with pernicious anemia in remission or with total gastrectomy more simply with the urinary excretion test than with the fecal recovery test, provided quantitativeness of absorption is not a consideration.

SUMMARY AND CONCLUSIONS

1. A method is described for carrying out the fecal elimination test for cobalt^{60}-labeled vitamin B_{12} absorption. The method does not require transfer of stool specimen from one container to another and each specimen can be counted within half an hour.

2. Results obtained in 42 patients in whom two or more tests were carried out under similar conditions indicate that the results are reproducible usually within less than 10 per cent variation. As a research tool for quantitative measurement of intestinal absorption of vitamin B_{12}, the method is therefore reliable.

3. The average recovery of 0.5 mcg. Co^{60}-B_{12} in 38 tests in 30 controls was 34 per cent, the greatest being 57 per cent and the lowest 5 per cent. The lowest recovery in 34 tests in patients with intrinsic factor deficiency (addisonian pernicious anemia or total gastrectomy) was 78 per cent with an average of 94 per cent. The test is thus reliable in the diagnosis of pernicious anemia. However, the greater simplicity of the urinary excretion test (Schilling) makes this preferable as a clinical test for that purpose.

4. The apparatus and technique described would provide a simple means of measuring fecal radioactivity of other gamma-emitting isotopes such as Fe^{59}.

We are indebted to Dr. Franz K. Bauer and Dr. Raymond L. Libby of the Radioisotope Service, Veterans Administration Center, Los Angeles, for advice and assistance.

REFERENCES

1. Heinle, R. W., Welch, A. D., Scharf, V., Meacham, G. C., and Prusoff, W. H.: Studies of Excretion (and Absorption) of Co^{60}-Labeled Vitamin B_{12} in Pernicious Anemia, Tr. A. Am. Physicians **65**: 214-222, 1952.
2. Schilling, R. F.: Intrinsic Factor Studies. II. The Effect of Gastric Juice on the Urinary Excretion of Radioactivity After the Oral Administration of Radioactive Vitamin B_{12}, J. LAB. & CLIN. MED. **42**: 860-866, 1953.
3. Glass, G. B., Boyd, L. J., Gellin, G. A., and Stephanson, L.: Uptake of Radioactive Vitamin B_{12} by the Liver in Humans: Test for Measurement of Intestinal Absorption of Vitamin B_{12} and Intrinsic Factor Activity, Arch. Biochem. **51**: 251-257, 1954.
4. Schilling, R. F., Clatanoff, D. V., and Korst, D. R.: Intrinsic Factor Studies. III. Further Observations, Utilizing the Urinary Radioactivity Test in Subjects With Achlorhydria, Pernicious Anemia, or a Total Gastrectomy, J. LAB. & CLIN. MED. **45**: 926-935, 1955.
5. Ellenbogen, L., Williams, W. L., Rabiner, S. F., and Lichtman, H. C.: An Improved Urinary Excretion Test as an Assay for Intrinsic Factor, Proc. Soc. Exper. Biol. & Med. **89**: 357-362, 1955.
6. Callender, S. T., and Evans, J. R.: The Urinary Excretion of Labeled Vitamin B_{12}, Clin. Sc. **14**: 2, 295-302, 1955.
7. Swendseid, M. E., Halsted, J. A., and Libby, R. L.: Excretion of $Cobalt^{60}$-Labeled Vitamin B_{12} After Total Gastrectomy, Proc. Soc. Exper. Biol. & Med. **83**: 226-228, 1953.
8. Halsted, J. A., Gasster, M., and Drenick, E. J.: Absorption of Radioactive Vitamin B_{12} After Total Gastrectomy: Relation to Macrocytic Anemia and to the Site of Origin of Castle's Intrinsic Factor, New England J. Med. **251**: 161-168, 1954.
9. Swendseid, M. E., Gasster, M., and Halsted, J. A.: Limits of Absorption of Orally Administered Vitamin B_{12}: Effect of Intrinsic Factor Sources, Proc. Soc. Exper. Biol. & Med. **86**: 834-836, 1954.
10. Halsted, J. A., Swendseid, M. E., Gasster, M., and Lewis, P. M.: The Effect of Antibiotics on Intestinal Absorption of Vitamin B_{12} in Disorders of the Gastrointestinal Tract, Am. J. Med. **19**: 297, 1955 (Abst.).
11. Halsted, J. A., Swendseid, M. E., Gasster, M., and Lewis, P. M.: Absorption of Radioactive Vitamin B_{12} in Disease of the Small Intestine: Relation to Macrocytic Anemia, Tr. Am. Clin. & Climatol. A. **66**: 18-36, 1954.
12. Callender, S. T., Turnbull, A., and Wakisaka, G.: Estimation of Intrinsic Factor of Castle by Use of Radioactive Vitamin B_{12}, Brit. M. J. **1**: 1-8, 1954.
13. Glass, G. B., Boyd, L. J., and Stephanson, L.: Intestinal Absorption of Vitamin B_{12} in Man, Science **120**: 74-75, 1954.
14. Callender, S. T., and Wans, J. R.: Observations on the Relationship of Intrinsic Factor to the Absorption of Labelled Vitamin B_{12} From the Intestine, Clin. Sc. **14**: 387-394, 1955.

STUDIES IN LEUKEMIA

V. Antigenic Differences Between Leukemic and Nonleukemic Red Blood Cells

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CHANGES induced by several viruses on red blood cells (RBC) in vitro are well known and have been demonstrated in influenza, vaccinia, variola, mumps, scrub typhus, Newcastle disease of fowls, fowl plague, swine influenza, pneumonia virus of mice, and ectromelia of mice.[1] These changes lead to agglutination, have no relationship to antigen-antibody reaction, are thought to be enzymatic in nature, and are known as Hirst's phenomenon.[2]

Viruses are also postulated to induce changes in RBC in vivo, leading to agglutination and hemolysis, as shown in atypical pneumonia[3-5] and in cases of hemolytic anemia in which a virus was isolated from the patient's blood.[6, 7] These changes are different from Hirst's phenomenon and occur on the basis of an antigen-antibody reaction demonstrable by the presence of various types of antierythrocytic immune bodies (cold agglutinins, cold hemolysins, and heterophile agglutinins) in the patients' blood. By means of antiserum prepared specifically for Newcastle disease virus (NDV)-treated RBC, Gardner and his co-workers[8] have detected antigenic modifications in RBC induced by NDV.

Three suggestions prompted investigation of antigenic differences between leukemic and nonleukemic red blood cells: The hypothesis that leukemia might be a virus disease,[9-13] detection of antigenic modifications induced in RBC by NDV, and the fact that anemia found in leukemia is at least partly due to an immunohemolytic mechanism.[14-18]

The present report deals with the preparation and use of an antiserum for leukemic RBC for the study of modified erythrocytes in human chronic lymphocytic leukemia (CLL) and AKR mouse leukemia. Red blood cells from patients with chronic lymphocytic leukemia were chosen because of the high incidence of hemolytic anemia in this disease.[16, 18]

MATERIALS AND METHODS

Immunization of Rabbits.—Two series of three rabbits each were used. One group was injected with pooled washed RBC from patients with CLL, the other group received pooled washed RBC from AKR mice with spontaneous leukemia. During washing, as much of the buffy coat was discarded as possible. Daily intravenous injections of washed RBC

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Aided by grants from American Blood Research Society, Illinois Division of American Cancer Society, and Damon Runyon Foundation.

Received for publication Jan. 12, 1956.

were given, beginning with 0.2 ml. and increasing the dose by 0.1 ml. daily until a dose of 1 ml. was reached. One week after the seventh injection, a sample of blood was taken from the ear vein to study the antibody response. If the titer of the serum was found unsatisfactory, another course of injections was given. By the end of the experiment four rabbits were dead; the remaining two, reported on in this study, had received three courses of injections over a six-week period. The animals were bled by cardiac puncture, and sera were collected, inactivated at 56° C. for one hour, and frozen until used.

Adsorption Technique.—Sera from rabbits injected with human CLL RBC, after inactivation, were adsorbed with increasing volumes of normal pooled RBC. The pool of RBC contained blood groups A, B, O, and AB obtained from normal blood-bank donors. Adsorptions were done at room temperature by the addition of packed, thrice-washed RBC to rabbit serum and the allowance of the tubes to stand for one hour with occasional agitation to mix cells and serum. The first adsorption was carried out with 1 ml. of serum to 1 ml. of packed RBC; the second adsorption used 1 ml. of serum to 2 ml. of packed RBC; and the third adsorption 1 ml. of serum to 2.5 ml. of packed RBC.

Sera from rabbits injected with leukemic AKR mouse RBC were adsorbed by the same procedure. A pool of normal nonleukemic AKR mouse RBC from six mice was used. In the three adsorptions, 0.4 ml. serum was added to 0.4 ml., 0.6 ml., and 0.8 ml. of packed RBC.

Standard Techniques for Titration.—Titration techniques, similar to those used in Rh antibody studies, were employed in testing the sera. Saline titration consisted of the making of serial twofold dilutions (1:1, 1:2, and so on) of serum with normal saline solution. Each tube contained 0.1 ml. of appropriate dilution. To each tube was added 0.05 ml. of 2 per cent washed RBC suspension in saline solution. Tubes were shaken well and allowed to stand at 37° C., 20° C., and 5° C. for two hours. Agglutination of the RBC was read macroscopically and microscopically. Serum albumin titration consisted of the making of the same serial dilutions except that normal rabbit serum was used as diluent. A 2 per cent suspension of RBC was made in 20 per cent bovine albumin and added to serum dilutions in each tube. Tubes were shaken well and incubated as above.

Papain titration was identical with the saline technique except that RBC were pretreated with papain and resuspended in saline solution.[19] Titrations using the enzyme-treated RBC may be read after incubation time of 30 minutes; however, for purposes of comparison, they were also read after two hours.

RESULTS

Rabbits given three courses of daily intravenous injections of human CLL RBC and leukemic AKR mouse RBC during six weeks built up anti-RBC agglutinins, as shown in Tables I and II. Highest titers were obtained at room temperature and at 5° C. in either saline solution or by use of enzyme-treated RBC in saline medium. Agglutinins were consistently of a higher titer for both human leukemic RBC and leukemic mouse RBC than for normal RBC.

Adsorption experiments demonstrated the failure of normal RBC of either mouse or human origin to adsorb completely the agglutinin for leukemic RBC from the serum. Use of human CLL RBC and leukemic AKR mouse RBC for adsorption of their respective sera completely removed the agglutinin for leukemic RBC.

Sera which had been adsorbed with normal RBC to the point where the only agglutinin remaining seemed to be one that reacted only with leukemic RBC were used as test sera against other cases of human leukemia and mouse leukemia. For this testing, only the saline titration with papain-treated RBC at 20° C. was used because it gave the highest titers. With use of adsorbed

TABLE I. RED CELL AGGLUTININ TITER IN SERUM OF CHRONIC LYMPHOCYTIC LEUKEMIA ERYTHROCYTE SENSITIZED RABBIT

RABBIT SERUM	TEMPERATURE	TECHNIQUE OF TITRATION FOR HUMAN RED CELL AGGLUTINATION					
		SALINE		SERUM ALBUMIN		PAPAIN SALINE	
		CHRONIC L. L. RED CELLS	POOLED NORMAL RED CELLS	CHRONIC L. L. RED CELLS	POOLED NORMAL RED CELLS	CHRONIC L. L. RED CELLS	POOLED NORMAL RED CELLS
Inactivated serum before adsorption	37° C.	1:512	1:64	1:64	1:32	1:4,096	1:512
	20° C.	1:4,096	1:512	1:64	1:16	1:4,096	1:512
	5° C.	1:2,048	1:256	1:32	1:4	1:2,048	1:512
After third adsorption	37° C.	1:2	Negative	Negative	Negative	1:8	Negative
	20° C.	1:16	Negative	1:2	Negative	1:16	Negative
	5° C.	1:32	Negative	Negative	Negative	1:16	Negative
Adsorption: 1 part serum to 3 parts packed CLL red cells	37° C.	Negative	Negative	Negative	Negative	Negative	Negative
	20° C.	Negative	Negative	Negative	Negative	Negative	Negative
	5° C.	Negative	Negative	Negative	Negative	Negative	Negative

L.L. = Lymphocytic leukemia.

TABLE II. RED CELL AGGLUTININ TITER IN SERUM OF LEUKEMIC AKR MOUSE ERYTHROCYTE SENSITIZED RABBIT

RABBIT SERUM	TEMPERATURE	TECHNIQUE OF TITRATION FOR MOUSE RED CELL AGGLUTINATION					
		SALINE		SERUM ALBUMIN		PAPAIN SALINE	
		LEUKEMIC AKR RED CELLS	POOLED NORMAL MOUSE RED CELLS	LEUKEMIC AKR RED CELLS	POOLED NORMAL MOUSE RED CELLS	LEUKEMIC AKR RED CELLS	POOLED NORMAL MOUSE RED CELLS
Inactivated serum before adsorption	37° C.	1:512	1:32	1:16	1:4	1:512	1:32
	20° C.	1:1,024	1:128	1:32	1:16	1:2,048	1:512
	5° C.	1:2,048	1:128	1:16	1:2	1:1,024	1:32
Second adsorption	37° C.	1:2	Negative	Negative	Negative	1:4	Negative
	20° C.	1:4	Negative	1:1	Negative	1:4	Negative
	5° C.	1:8	1:1	Negative	Negative	1:8	Negative
Adsorption: 1 part serum to 2 parts packed leukemic AKR mouse red cells	37° C.	Negative	Negative	Negative	Negative	Negative	Negative
	20° C.	Negative	Negative	Negative	Negative	Negative	Negative
	5° C.	Negative	Negative	Negative	Negative	Negative	Negative

serum of the rabbit injected with human CLL RBC, a series of 14 cases of leukemia and 20 normal cases was studied. The RBC of six cases of CLL, five cases of chronic myelogenous leukemia, two cases of monocytic leukemia, and one case of acute myeloblastic leukemia all gave positive hemagglutination tests. The RBC of 20 normal cases, including all major blood groups, gave negative results. A group of six leukemic AKR mice gave positive reactions, whereas six young nonleukemic AKR mice gave negative reactions. Three mice of the DBA strain and four mice of the white Swiss strain also gave negative reactions. These studies are summarized in Table III.

With use of adsorbed serum of the rabbit injected with leukemic AKR mouse RBC, positive hemagglutinations were obtained with six leukemic AKR mice tested. Negative results were obtained with RBC from six young nonleukemic AKR mice, six white Swiss mice, and four DBA strain mice. Human

RBC from two cases of CLL gave positive results. Three cases of human CLL, three cases of chronic myelogenous leukemia, and two cases of monocytic leukemia all gave negative results. These results are recorded in Table IV.

TABLE III. ADSORBED SERUM OF RABBIT INJECTED WITH HUMAN CHRONIC LYMPHOCYTIC LEUKEMIA RED CELLS

TYPE OF RED CELLS TESTED (ENZYME TREATED)	NUMBER OF CASES	RANGE IN TITER OF RED CELL AGGLUTINATION (SALINE TITRATION AT 20° C.)
Chronic lymphocytic leukemia	6	1:4-1:16
Chronic myelogenous leukemia	5	1:2-1:8
Monocytic leukemia	2	1:2-1:4
Acute myeloblastic leukemia	1	1:4
Normal red cells of all blood groups	20	Negative
Leukemic AKR mouse red cells	6	1:2-1:8
Nonleukemic AKR mouse red cells	6	Negative
DBA strain mouse red cells	3	Negative
White Swiss strain mouse red cells	4	Negative

TABLE IV. ADSORBED SERUM OF RABBIT INJECTED WITH LEUKEMIC AKR MOUSE RED CELLS

TYPE OF RED CELLS TESTED (ENZYME TREATED)	NUMBER OF CASES	RANGE IN TITER OF RED CELL AGGLUTINATION (SALINE TITRATION AT 20° C.)
Leukemic AKR mouse red cells	6	1:2-1:4
Nonleukemic AKR mouse red cells	6	Negative
White Swiss strain mouse red cells	6	Negative
DBA strain mouse red cells	4	Negative
Chronic lymphocytic leukemia human red cells	2	1:2-1:4
Chronic lymphocytic leukemia human red cells	3	Negative
Chronic myelogenous leukemia human red cells	3	Negative
Monocytic leukemia human red cells	2	Negative

Trypsin-treated RBC gave similar results to papain-treated RBC. Normal RBC treated with trypsin gave negative results. Several different specimens of normal noninjected rabbit serum were used as a negative control for testing against cells that gave positive results with immunized rabbit sera. None of the normal rabbit sera gave positive agglutination reactions with RBC of leukemic patients or of leukemic AKR mice.

DISCUSSION

Our experiments demonstrate an antigenic modification of the RBC in both human CLL and in spontaneous AKR mouse leukemia. The highest agglutinin titers were obtained with papain-treated and saline-suspended RBC at 20° C. and 5° C. When enzyme-treated RBC are used as test cells, it is possible to detect univalent as well as bivalent antibodies with a high degree of specificity and sensitivity. We may assume that in our serum a bivalent antibody is present, although we cannot be sure whether a univalent antibody also exists. The high titers obtained at 5° C., as well as at 20° C., indicate the presence of agglutinins having a wide thermal amplitude, partly of the cold agglutinin type. The findings, showing the presence of specific bivalent antibodies, are consistent with reports of the presence of an autoimmune hemolytic disease in malignant lymphocytic diseases, detected by different methods, but mainly by the red cell survival technique.[14-18]

An interesting observation is the cross-reaction between human and mouse leukemia and between human myelogenous and lymphocytic leukemia. The reason for this cross-reaction must be a similar antigenic modification of the RBC which may be secondary to a common agent or at least antigenically similar causative agent.[13] Another explanation might be that an abnormal globulin is elaborated by the diseased or deranged reticuloendothelial system,[22] which coats the RBC so that serum which we are using is acting as an anti-globulin serum. In this connection it should be mentioned parenthetically that hemolytic anemia of the autoimmune type is rarely found in other than lymphocytic leukemias.[20, 21] It may be simply that laboratory tests now available fail to detect mild degrees of autoimmune hemolytic anemias. On the other hand, the causative agent may actually differ in different forms of leukemia. Further studies with antigens produced for the RBC of different leukemias should help clarify this question. Previous studies in our laboratories have suggested a general similarity of the antigens of various leukemias, but with subtle differences.[13]

It is interesting to speculate on the nature of the antigenic modification. But the most important question is whether or not the modifications here demonstrated are similar to those induced by viruses or viral agents. The presence of bivalent antibodies leads us to believe that specific receptors created on the RBC are of a virus nature because of the close similarity between our findings and those obtained by use of NDV-treated RBC.[8]

The presence of T agglutinogens was excluded as an explanation when normal RBC treated with trypsin failed to show any agglutination by the rabbit serum.

SUMMARY

1. Specific antisera were prepared for RBC obtained in cases of human CLL and AKR mouse spontaneous leukemia.

2. Antigenic modifications were detected in the RBC in both conditions.

3. A cross-agglutination was demonstrable among various human leukemias as well as between human and mouse leukemia.

4. The nature and the possible viral etiology of the RBC modification are discussed.

REFERENCES

1. Rivers, T. M.: Viral and Rickettsial Infections of Man, Philadelphia, 1948, J. B. Lippincott Co., p. 77.
2. Hirst, G. K.: The Agglutination of Red Cells by Allantoic Fluid of Chick Embryos Infected With Influenza Virus, Science **94**: 22, 1941.
3. Aaron, R. S.: Hemolytic Anemia in Viral Pneumonia With High Cold Agglutinin Titer, A. M. J. Arch. Int. Med. **89**: 293, 1952.
4. Peterson, O. L., Ham, T. H., and Finland, M.: Cold Agglutinins (Autohemagglutinins) in Primary Atypical Pneumonia, Science **97**: 167, 1943.
5. Turner, J. C.: Development of Cold Agglutinins in Atypical Pneumonia, Nature **151**: 419, 1943.
6. Moolten, S. E., and Clark, E.: Viremia in Acute Hemolytic Anemia and in Auto-Hemagglutination. Report of a Case and Review of the Literature With Special Reference to the Virus of Newcastle Disease, A. M. A. Arch. Int. Med. **89**: 270, 1952.
7. Moolten, S. E., and Clark, E.: The Red Blood Cell as a Vehicle of Virus Transport. II. Role of Blood Borne Viruses in Autohemagglutination and in Hemolytic Anemia, Tr. New York Acad. Sc. **14**: 235, 1952.

8. Gardner, E., Jr., Wallace, J. H., Dodd, M. C., and Claude-Starr, Wright: Antigenically Modified Red Cells in Chickens Infected With Newcastle Disease, Proc. Soc. Exper. Biol. & Med. **87**: 253, 1954.
9. Gross, L.: The "Vertical" Transmission of Mouse Mammary Carcinoma and Chicken Leukemia. Its Possible Implication for Human Pathology, Cancer **4**: 626, 1951.
10. Gross, L.: Transmissible Mouse Leukemia. Biological Properties of the Mouse Leukemia Agent. Ciba Symposium on Leukemia Research, London, Nov. 16-19, 1953.
11. Gross, L.: Is Leukemia Caused by a Transmissible Virus? A Working Hypothesis, Blood **9**: 557, 1954.
12. Magrassi, F., Leonardi, G., Negroni, G., and Tolu, A.: Experimental Study on the Etiology of Human Leukemia, Acta haemat. **6**: 38, 1951.
13. Schwartz, S. O., Schoolman, H. M., and Spurrier, W.: Studies in Leukemia. III. Agglutination of Human Leukemic Cells by Guinea Pig Serum, J. LAB. & CLIN. MED. **46**: 709, 1955.
14. Young, L. E., Miller, G., and Christian, R. M.: Clinical and Laboratory Observations on Autoimmune Hemolytic Disease, A. M. A. Ann. Int. Med. **35**: 507, 1951.
15. Ross, J. F., Crockett, C. L., Jr., and Emerson, C. P.: The Mechanism of Anemia in Leukemia and Malignant Lymphoma, Proc. Am. Soc. Clin. Invest. **30**: 130, 1951.
16. Brown, G. M., Elliott, S. M., and Young, W. A.: The Hemolytic Factor in the Anemia of Lymphatic Leukemia, J. Clin. Invest. **30**: 130, 1951.
17. Berlin, R.: Red Cell Survival Studies in Normal and Leukaemic Subjects, Acta med. scandinav. (Suppl. 252) **139**: 1-141, 1951.
18. Rosenthal, M. C., Pisciotta, A. V., Komninos, Z. D., Goldenberg, H., and Dameshek, W.: The Autoimmune Hemolytic Anemia of Malignant Lymphocytic Disease, Blood **10**: 197, 1955.
19. Kuhns, W. H., and Bailey, A.: Use of Red Cells Modified by Papain for Detection of Rh Antibodies, Am. J. Clin. Path. **20**: 1067, 1950.
20. Stats, D., Rosenthal, N., and Wassermann, L. R.: Hemolytic Anemia Associated With Malignant Disease, Am. J. Clin. Path. **17**: 585, 1947.
21. Aubert, A., and Brendemoen, O. J.: Acquired Hemolytic Anemia and Lymphoblastoma, Scandinav. J. Clin. & Lab. Invest. **1**: 95, 1949.
22. Rundles, R. W., Coonrad, E. V., and Arends, T.: Serum Proteins in Leukemia, Am. J. Med. **16**: 842, 1954.

STUDIES ON HEPATOLENTICULAR DEGENERATION

Copper as the Causative Agent in the Degeneration and Dysfunction of Electric Tissue

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COPPER deposition in the brain, liver, kidneys, and muscular tissues, as well as anatomical changes of the brain, in patients with hepatolenticular degeneration has been demonstrated by various workers.[1-4] It has been assumed that such copper deposits are the eventual cause of tissue degeneration, which in turn brings about the clinical manifestations. Treatment of patients with hepatolenticular degeneration employing such agents as dimercaprol, which function by removing some of the excess copper stores, has been successful in bringing about clinical improvement.[5, 6] Since clinical improvement does follow the removal of some of the stored copper, this effect can be taken as a very good indication of the mediation by copper in the clinical reaction of the patient. For this reason it appears safe to assume that the clinical manifestations (i.e., the neurological signs more specifically) are originally induced by chemical reactions involving copper rather than by brain tissue degeneration per se. There is no information now available in regard to the reaction mechanisms involving copper in this disease. The present investigation was carried out in an attempt to shed some light on the particular mechanism in which copper stores affect the general metabolism of nervous tissue and the interference of copper on nerve conduction in Wilson's patients.

Although there are many reports of inhibitory or even activating properties of various heavy metals in general and copper in particular on enzyme systems, those studies were carried out with free metal ions.[7-10] In the case of Wilson's disease, copper accumulates in the brain and other tissues not as ionic copper but in combination with chelating agents of one kind or another. These chelating agents are most probably peptides or proteins.[1, 11] For this reason it seemed appropriate to study the effect of various copper chelates upon partly purified enzyme systems.

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This work was supported by Grant (B-543C), National Institutes of Health, Public Health Services.
Received for publication Jan. 17, 1956.
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Three enzyme systems were used in this investigation, alkaline serum phosphatase, acid serum phosphatase, and myosin B-adenosinetriphosphatase. These three systems were selected because of the reported effect of copper upon adenosinetriphosphatase, and, moreover, these enzymes, especially adenosinetriphosphatase, assume a key role in human metabolism. The influence of copper upon nerve conduction was felt to be an integral part of this investigation and for this the sciatic nerve-gastrocnemius muscle of the frog was employed.

MATERIALS AND METHODS

Enzyme.—Fresh serum was used as a source of acid and alkaline phosphatase from a patient with Wilson's disease and from control subjects. On the basis of the initial studies no attempt was made to purify these enzymes. Myosin B-adenosinetriphosphatase was obtained from rat muscle and fresh pig brain according to the method of Greenstein and Edsall.[12] Enzyme activity was measured according to the method of DuBois and Potter.[9] Adenosinetriphosphate, 99 per cent,* was used as substrate. The copper chelates were made up by mixing $CuSO_4$ with solutions of the given amino acids in the given molar ratios using a fixed copper concentration of 8×10^{-5} M in the incubation mixture. Optical densities were measured at 660 mμ on a Beckman B spectrophotometer. Alkaline and acid phosphatase activity was measured according to a modification of the method of Bodansky.[13]

Nerve-Muscle Preparation.—The experiments with isolated sciatic nerve-gastrocnemius muscle were carried out as follows: The animals were pithed and the sciatic nerve was freed from the surrounding connective tissue as far medially as the emergence of the nerve rootlets from the vertebral column. The gastrocnemius muscle was then freed in its distal portion but left attached to the femur proximally. As the final step, the nerve roots were severed as close to the vertebral column as possible. An attempt was made to strip the vasa nervorum from the perineurium to help insure easier diffusion of the solutions into the nerve. During the experiments the nerve-muscle preparation was kept in a moisture-saturated atmosphere. At a point midway between the stimulating electrodes and the muscle, the nerve was immersed in a paraffin-coated porcelain boot for a distance of about 8 mm. Care was taken to prevent the copper and other solutions from running beyond the confines of this boot. Stimulating current was provided by the usual inductorium.

Acetylcholine† was used in freshly prepared dilutions of 1:10,000, unless otherwise stated, with cold-blooded Ringer's solution as diluent.

The sodium salt of 1, 2-naphthoquinone-4-sulfonic acid for inhibition of choline acetylase was used in freshly prepared dilutions, as indicated with cold-blooded Ringer's solution as diluent.

RESULTS

The activity of serum, alkaline, and acid phosphatase from a patient with Wilson's disease and two control subjects was measured. No significant difference in activities could be observed either with the serum alone or with $CuSO_4$ at 2×10^{-7}, 2×10^{-6}, 2×10^{-5}, and 2×10^{-4} M final concentration in the incubation mixture.

The effect of copper and copper chelates on the activity of myosin B-adenosinetriphosphatase was studied. It was necessary however to select a $CuSO_4$ concentration which would give the greatest accuracy in a determination of the inhibitory or stimulatory effect of copper upon the above-mentioned enzyme. Various $CuSO_4$ concentrations were used and a final concentration of 8×10^{-5} M

*Adenosinetriphosphate and amino acids were procured from Nutritional Biochemicals Corporation, Cleveland, Ohio.

†Acetylcholine was obtained from Eastman Kodak Company.

selected. At this level there was 60 per cent inhibition of adenosinetriphosphatase activity and by employing 0.2 ml. of the enzyme preparation a slight alteration of activity was accompanied by a great difference in phosphorus liberation. In spite of all these precautions and all efforts to standardize conditions, quite contradictory results in successive experiments with the same enzyme preparations were obtained.

Myosin B-adenosinetriphosphatase from rat muscle and pig brain (the latter both dialyzed [16 hr.] and undialyzed) behaved in the same fashion. The KCL concentration was held at 0.6 M. KCL is known to affect the action of ethylene diaminetetraacetic acid upon adenosinetriphosphatase very markedly, an inhibition being observed at low KCL concentrations and an acceleration at high levels.[14-18]

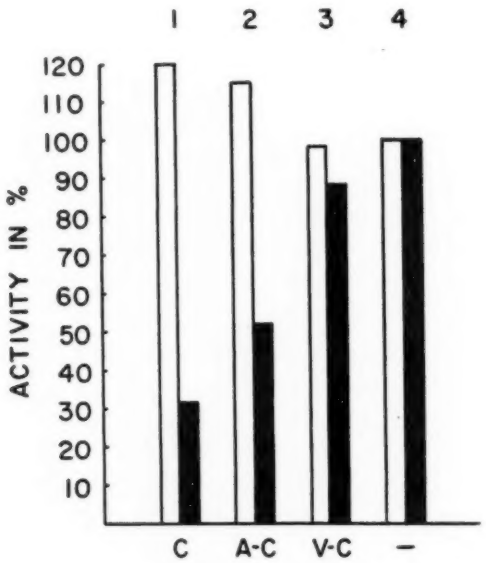

Fig. 1.—The effect of $CuSO_4$, arginine, and Versene-Cu chelates upon myosin B-adenosinetriphosphatase. *1*, $CuSO_4$ 8×10^{-5} M, *2*, arginine-Cu 8×10^{-5} M, *3*, Versene-Cu 8×10^{-5} M, *4*, myosin B only. Open bars represent one experiment where stimulation with $CuSO_4$ was observed. Black bars represent values of one typical experiment showing inhibition with $CuSO_4$ and the copper chelates.

In the majority of determinations ionic copper exerted more of an inhibition of the adenosinetriphosphatase than the copper chelates. This is shown in Fig. 1 (solid bars), where the height of the bars represents the amount of dephosphorylation of adenosinetriphosphate due to adenosinetriphosphatase in the presence and absence of copper and various copper chelates. The amount of dephosphorylation with adenosinetriphosphatase alone is represented as 100 per cent. Dephosphorylation in presence of copper and the copper complexes is expressed in per cent of this control value. In those cases where ionic copper showed only little inhibition of the enzyme activity, a more pronounced inhibition was noted on the part of the arginine-copper chelate. In two instances ionic copper produced a definite stimulation of adenosinetriphosphatase activity.

As illustrated in Fig. 2 definite inhibition was obtained with the arginine-copper chelates in all cases regardless of the particular arginine-copper ratio used. However, when a stronger inhibition was induced by the one-part arginine to two-parts copper complex, less inhibition was observed on the part of the two-

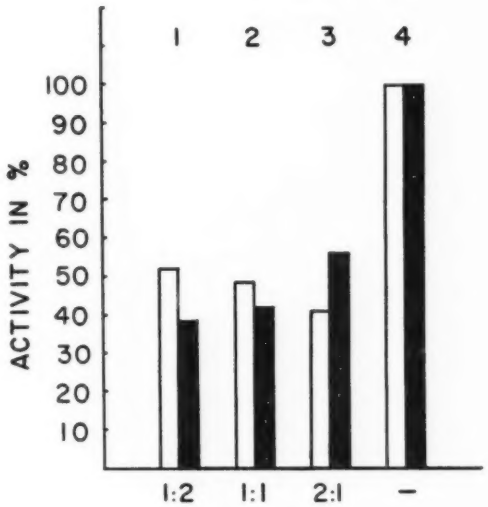

Fig. 2.—The effect of altering the arginine-copper ration on the inhibiting action upon myosin B. Open bars and black bars give values for one representative experiment, each showing the two different trends observed. Cu concentration in all cases 8×10^{-5} M. *1*, arginine – Cu = 1:2, *2*, arginine – Cu = 1:1, *3*, arginine – Cu = 2:1, *4*, myosin B only.

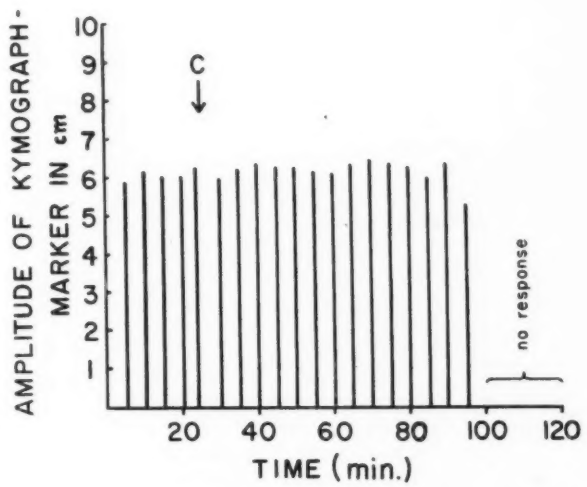

Fig. 3.—Effect of Cu on the isolated frog sciatic nerve-gastrocnemius muscle preparation. At arrow 10^{-3} M $CuSO_4$ solution added to the boot.

parts arginine to one-part copper complex, and vice versa, the one to one ratio always exhibiting intermediate effects. In general the ratio of two-parts arginine to one-part copper behaves similarly to the Versene copper complex,

Table I presents a comparison of various inhibitors acting on the sciatic-gastrocnemius nerve-muscle preparation of the frog and the effect of acetylcholine upon these inhibitors.

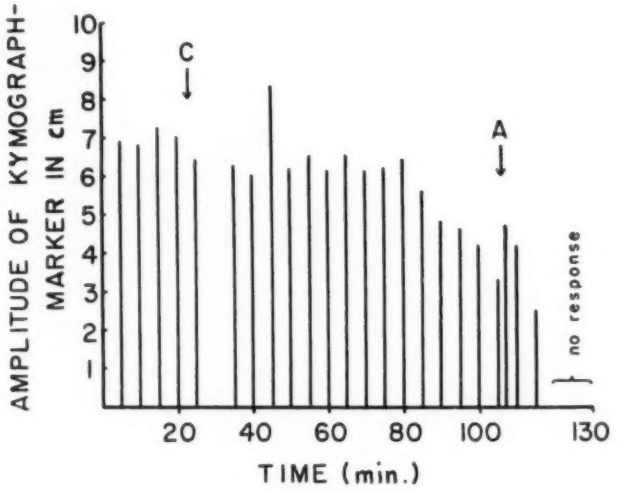

Fig. 4.—Effect of acetylcholine on the Cu inhibition. Same system as in Fig. 3. At *C*, 10^{-5} M $CuSO_4$ added to the boot. At *A*, boot washed and acetylcholine (1:5,000) added.

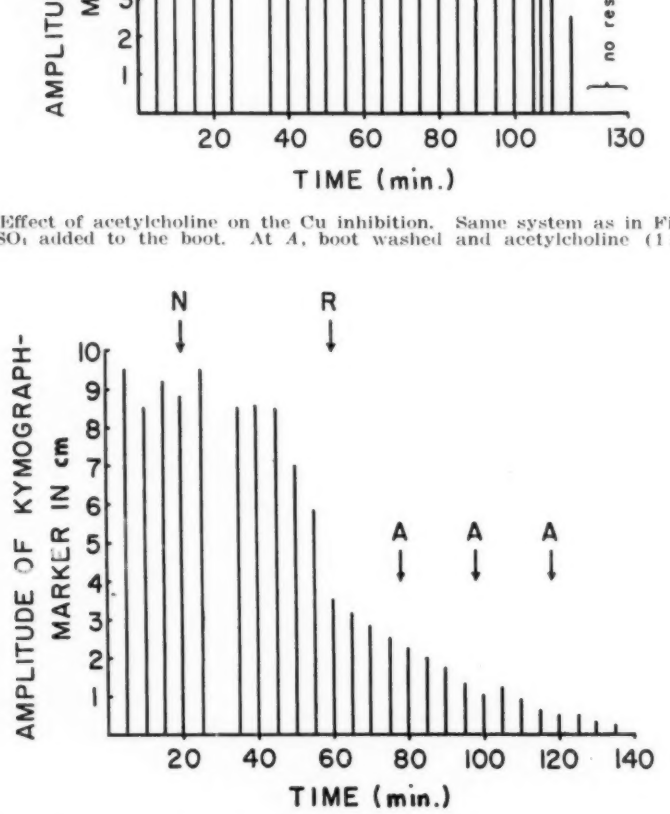

Fig. 5.—Effect of acetycholine on the naphtoquinone sulfonic acid inhibition of the isolated frog sciatic nerve-gastrocnemius muscle preparation. At *N*, 10^{-6} M naphtoquinone sulfonic acid to the boot. At *R*, boot washed with Ringer's solution. At *A*, acetylcholine (1:5,000) added.

Fig. 3 serves to show a typical extinction pattern produced by the inhibitors without an attempt to restore activity by acetylcholine. The observable decrease in amplitude is quite precipitous at 75 minutes, with only a slight decrease as a forewarning after 70 minutes of nerve intoxication. Figs. 4, 5, and 6 are

representative of the response changes as recorded on the kymograph, which were produced by the inhibiting substances and the reactions to acetylcholine after inhibition was induced.

TABLE I. THE EFFECT OF COPPER AND NAPHTOQUINONE SULFONIC ACID UPON THE SCIATIC NERVE PREPARATION OF THE FROG

INHIBITOR	$CuSO_4$ 5×10^{-4} M	$CuSO_4$ 10^{-4} M	$CuSO_4$ 10^{-5} M	NAPH. QU. 3×10^{-5} M	NAPH. QU. 10^{-6} M
Time and degree of inhibition	15 Min. 80%	105 Min. 100%	85 Min. 50%	50 Min. 30%	45 Min. 50%
Material used for reversal and observed effect	Acetylcholine No response	Choline acetylase No response	Acetylcholine reversed from 70% to 50%	Acetylcholine Definite return of response	Acetylcholine Decreased rate of inhibition

In Fig. 4, washing the nerve with Ringer's solution after 40 minutes tends to slow the process of inhibition, while the second addition of acetylcholine after 80 minutes of intoxication is followed by a temporary increase in the amplitude of the response within 10 minutes. When acetylcholine was added a third time after 105 minutes, the tendency for a straight-line extinction was interrupted, although no increase in amplitude resulted. This was not the case when the nerve was washed with Ringer's solution after 120 minutes and the response curve extended on to the anticipated complete cessation of activity.

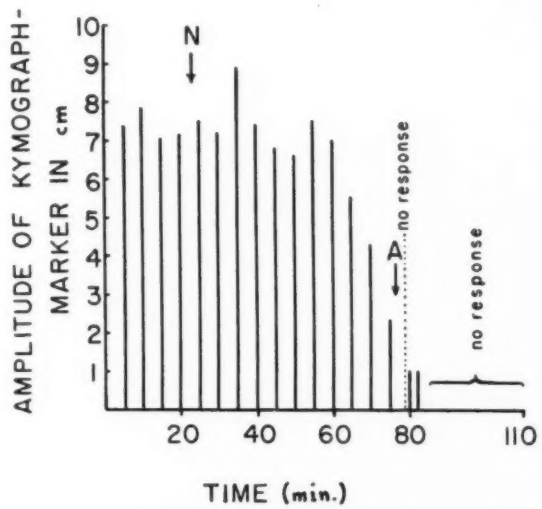

Fig. 6.—The same system as in Fig. 4, but an inhibitor concentration of 3×10^{-5} M naphtoquinone sulfonic acid was used.

In Fig. 5 we see a partial although temporary recovery of activity following the addition of acetylcholine even though complete inhibition of activity had occurred. Fig. 6 also shows a partial and temporary reversal of inhibition with the removal of copper and the addition of acetylcholine after 85 minutes of intoxication.

DISCUSSION

In an attempt to determine the effect of copper upon conductive tissues, a search of the literature reveals that the amount of copper employed in the classic experiments was rather high. Bailey[7] used about 6×10^{-3} M copper as an inhibitor of adenosinetriphosphatase, and Lipman[19] used 0.64 mg. per milliliter to determine the effect upon glycolysis of intact muscle. In the present study much lower concentrations were employed in order to approach more physiologic conditions.

The serum phosphatase in both Wilson's patients and controlled subjects appeared not to be influenced by the copper concentration employed. It can therefore be concluded that in patients with Wilson's disease, the serum phosphatases were unaffected by copper, as the serum copper concentration in these patients is well below the concentrations used in these in vitro experiments.

The dephosphorylation mechanism of adenosinetriphosphate proved to be very sensitive to copper not only at the level of 8×10^{-5} M final concentration but also at 8×10^{-6} M. In addition to its sensitivity to free ionic copper, this system was also shown to be sensitive to copper combined with complexing agents, the latter being a far more physiologic condition that is the free ionic copper. However, on the basis of the experimental evidence obtained it cannot be stated positively that under physiologic conditions in vivo inhibition would take place. In fact under certain circumstances it may be entirely possible that a stimulatory effect could be produced by the copper complexes. The degree of inhibition observed in these experiments, however, would be sufficient to explain a disturbance of metabolism in tissues loaded with copper and the ensuing tissue degeneration, such as is the case in brain tissues of patients afflicted with Wilson's disease. Copper stores in the body would be expected to influence a multitude of systems, and most likely enzyme systems containing reactive sulfhydryl groups[18] would be concerned. In the first part of this investigation it was shown that the systems concerned with the dephosphorylation of adenosinetriphosphate are so affected. The interaction of copper with these particular enzymes offers one explanation for this degenerative process going on in the brain of patients afflicted with hepatolenticular degeneration. On the basis of findings[5, 6] that the reduction of copper stores brings about improvement of the clinical symptoms, it will have to be assumed that the effect of copper is at least twofold. First, it brings about the slow process of degeneration of various parts of the brain, and, second, there is an interaction with the mechanism of neuronal conduction. If the clinical picture were to depend solely on the degree of degeneration of the brain, relief of the symptoms by the removal of copper would not be expected.

More direct evidence of a link between copper and nerve conductivity was demonstrated by the use of the muscle preparation. As can be seen from Fig. 3, the addition of copper to the nerve is followed by a considerable lag period which in turn is followed by a rapid drop in conductivity. The most likely explanation for this seems to be that the enzyme systems affected by copper are present in large excess. Only when the active enzyme concentration is reduced to a suboptimal level is a change in conductivity observed. However, the rate of variable

diffusion in large nerve bundles makes quantitative evaluation difficult. On the basis of the observed data it seems safe to assume that hardly more than 30 per cent of the affected enzyme component is necessary for optimum nerve activity. This is based on experiments with the lowest concentration of inhibitors employed. One of the direct interactions with nerve conductivity may very well be through the adenosinetriphosphate system, since this substance is required for the acetylation of choline to acetylcholine.[15] The enzyme which catalyzes this reaction, choline acetylase, has also been shown to be sensitive to copper[15] and naphtoquinone sulfonic acid (a more specific inhibitor of choline acetylase).[16, 17] If choline acetylase were the foremost affected system, a complete reversal of the copper inhibition by the addition of acetylcholine should take place. This would also be true if through the interaction of copper the amount of adenosinetriphosphate present would be the limiting factor. If the systems related to nerve conduction beyond the point of choline acetylation would be affected, no reversal should take place through the addition of acetylcholine. As a third possibility, a different sensitivity toward copper by more than one affected enzyme system or a different rate of destruction of such systems would manifest itself through at least a partial and only temporary reversal of inhibition by the addition of acetylcholine. This is what has actually been observed.

Figs. 4, 5, and 6 illustrate that acetylcholine produces a partial and temporary reversal of the inhibition of nerve conductivity after poisoning with copper and naphtoquinone sulfonic acid. These experiments constitute evidence that a copper-sensitive system mediates the process of nerve conduction beyond the point of choline acetylation.

SUMMARY

An effect of copper upon the dephosphorylation system of adenosinetriphosphate of muscle and brain tissue preparations was observed. Effective copper concentrations were as low as 8×10^{-6} M. In all but two experiments an inhibition was observed. In these two experiments an actual stimulation was seen. Copper chelates with arginine and Versene showed intermediate effects. No effect on alkaline and acid serum phosphatase was found with copper concentrations of 2×10^{-4} M, 2×10^{-5} M, 2×10^{-6} M, and 2×10^{-7} M final concentrations.

The conductivity of the frog sciatic nerve was inhibited with copper 10^{-5} M and naphtoquinone sulfonic acid 10^{-6} M. Both inhibitions were partially and temporarily reversible by acetylcholine.

REFERENCES

1. Gilsanz, V., Segovia, J. M., and Castro Mendoza, H.: Contribution to Study of Wilson's Disease, A. M. A. Arch. Int. Med. **95**: 727, 1955.
2. Cumings, J. N.: The Copper and Iron Content of Brain and Liver in the Normal and in Hepato-Lenticular Degeneration, Brain **71**: 410-415, 1948.
3. Glazebrook, A. J.: Wilson's Disease, Edinburgh M. J. **52**: 83, 1945.
4. Haurowitz, F.: Über eine Anomalie des Kupferstoffwechsels, Ztschr. f. Physiol., Chem. **190**: 72, 1930.

5. Denny-Brown, D., and Porter, H.: The Effect of BAL (2, 3-dimercaptopropanol) on Hepatolenticular Degeneration (Wilson's Disease), New England J. Med. **245:** 917, 1951.
6. Raskin, N., and MacKenzie, J. M.: Hepatolenticular Degeneration: Clinical and Pathological Report of Case Treated with BAL, J. Nerv. & Ment. Dis. **120:** 176, 1954.
7. Bailey, K.: Myosin and Adenosine Triphosphatase, Biochem. J. **36:** 121, 1942.
8. Kalckar, H. M.: Adenylpyrophosphatase and Myokinase, J. Biol. Chem. **153:** 355, 1944.
9. DuBois, K. P., and Potter, V. R.: The Essay of Animal Tissues for Respiratory Enzymes, J. Biol. Chem. **150:** 185, 1943.
10. Singher, H. C., and Meister, A.: The Adenosinetriphosphatase Activity of Myosin Preparations, J. Biol. Chem. **159:** 491, 1945.
11. Hansl, N., Hyman, I., and Zimdahl, W. T.: Copper Absorption Mechanisms in the Lower Intestine; Their Relation to Hepatolenticular Degeneration. Unpublished data.
12. Greenstein, J. P., and Edsall, J. T.: The Effect of Denaturing Agents on Myosin. I. Sulfhydryl Groups as Estimated by Porphyrindin Titration, J. Biol. Chem. **133:** 396, 1940.
13. Hawk, P. B., Oser, B. L., and Summerson, W. H.: Pract. Physiol. Chem. p. 637-8, based on Shinowara, G. Y., Jones, L. M., and Reinhart, H. L.: The Estimation of Serum Inorganic Phosphate and "Acid" and "Alkaline Phosphatase" Activity, J. Biol. Chem. **142:** 921, 1942.
14. Gross, M.: Effect of Ethylenediaminetetraacetic Acid (EDTA) on Adenosinetriphosphatase Activity, Science **118:** 218, 1953.
15. Bowen, W. J., and Kerwin, T. D.: Ethylenediaminetetraacetic Acid (EDTA) on ATP-ase Activity of Myosin, Fed. Proc. **13:** 16, 1954.
16. Friess, E. T.: Effect of a Chelating Agent on Myosin ATPase, Fed. Proc. **13:** 49, 1954.
17. Friess, E. T.: The Effect of a Chelating Agent on Myosin ATPase, Arch. Biochem. **51:** 17, 1954.
18. Bowen, W. J., and Kerwin, T. D.: A Study of the Effects of Ethylenediaminetetraacetic Acid on Myosin Adenosinetriphosphatase, J. Biol. Chem. **211:** 237, 1954.
19. Lipman, F.: Über eine Aktivierung der Glykolyse durch Kupfer, Biochem. Ztschr. **268:** 314, 1935.
20. Singer, T. P., and Barron, E. S. G.: The Effect of Sulfhydryl Reagents on Adenosinetriphosphatase Activity of Myosin, Proc. Soc. Exper. Biol. & Med. **56:** 920, 1944.
21. Nachmansohn, D., and Wilson, I. B.: The Enzyme Hydrolysis and Synthesis of Acetylcholine, Advances Enzymol. **12:** 359, 1951.
22. Nachmansohn, D., and Berman, M.: Studies on Choline Acetylase; on the Preparation of the Coenzyme and Its Effect on the Enzyme, J. Biol. Chem. **165:** 551-563, 1946.
23. Nachmansohn, D., and Weiss, M. S.: Studies on Choline Acetylase; Effect of Citric Acid, J. Biol. Chem. **172:** 677-687, 1948.

THE STABILITY OF THE VIRAL POTENCY OF SMALLPOX VACCINE WHEN PREPARED IN THE GLOBULIN AND OTHER PROTEIN FRACTIONS OF CALF PLASMA, DEHYDRATED, AND STORED AT TROPICAL TEMPERATURES

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AN IDEAL smallpox vaccine is one which is still suitable for human immunization by fulfilling the requirements of infectivity and sterility of the National Institute of Health[19] after an exposure to tropical temperatures of 30 days or longer.

The glycerinated vaccines in use today become unfit for human vaccination in a matter of days when exposed to temperatures above 5° C.[2]

The need for a smallpox vaccine that tolerates high temperatures reasonably well has been recognized especially wherever large emergency stock-piles of it are stored at low temperatures for the protection of populations of large cities against smallpox epidemics. Wherever such stock-piles are amassed, the fear exists that a power supply failure to the storage freezers because of fire, hurricane, disaster, etc., would cause rapid destruction to the vaccine supply at a time when it might be needed most.

Efforts have been made through the years by many workers[3-16] to develop such a vaccine, and much valuable information has been compiled.

A report is made here of the comparative protective value to high temperatures afforded dehydrated smallpox virus by calf globulin and other distinct fractions of protein derived and isolated by chemical means from calf plasma.

MATERIALS AND METHODS

I. *Materials.*—Preparation of the protein fractions of calf plasma used in the tests.

1. *Fibrin fraction:* The fibrin obtained by centrifuging calf plasma was washed four times in fresh volumes of sterile distilled water. The sediment was brought to a final volume containing 11.9 Gm. of protein per 100 ml.; 1 per cent NaCl, 0.6875 per cent phenol, and 1 per cent ether were added. The reaction was adjusted to pH 7.1.

2. *Globulin, not heated:* Calf plasma was centrifuged to remove fibrin.* To the clear material was added an equal volume of a saturated solution of $(NH_4)_2SO_4$ to 50 per cent saturation. The resulting precipitate was collected by filtration, dried between sheets of absorbent paper, and dialyzed against distilled water at 3° C. until the $SO_4^=$ content was less than 1 per cent. Enough NaCl, phenol, and ether were added to give respective final concentrations of 1 per cent, 0.6875 per cent, and 1 per cent. The reaction was adjusted to pH 7.1.† The total protein content of the globulin was 15.1 Gm. per 100 ml.

From The Bureau of Laboratories, New York City Department of Health, Foot of East 15th Street, New York, N. Y.
Received for publication Jan. 1, 1956.
*The fibrin collected was used to prepare fibrin fraction 1.
†All pH measurements were made with a Beckman apparatus.

3. *Globulin, heated:* Calf plasma was defibrinated as above* and heated at 57° C. for one-half hour. The globulin was then extracted and concentrated as described above. The same concentrations of reagents were added and the reaction was adjusted to pH 7.1. The total protein content of this product was 14.4 Gm. per 100 ml.

4. *Euglobulin fraction:* Calf plasma was centrifuged and filtered until perfectly clear. It was precipitated to 30 per cent saturation with saturated $(NH_4)_2SO_4$ solution. The precipitate was collected by filtration,† semidried, and dialyzed as described above. The same concentrations of reagents were added and it was adjusted to pH 7.1. The total protein content was 8.42 Gm. per 100 ml.‡

5. *Pseudoglobulin fraction:* The clear filtrate from the euglobulin precipitation, described above, was treated with more saturated $(NH_4)_2SO_4$ solution until 50 per cent saturation was obtained. The precipitate was collected by filtration,§ semidried, and dialyzed as before. One per cent NaCl, 0.6875 per cent phenol, and 1 per cent ether were added and the reaction adjusted to pH 7.1. The total protein content was 16.1 Gm. per 100 ml.

6. *Albumin fraction:* The clear filtrate from the pseudoglobulin precipitation was treated with glacial acetic acid to pH 4.3. The resulting albumin precipitate was filtered. The semidried precipitate was sprinkled with $NaCO_3$ powder to neutralize the acid reaction during dialysis which was performed as before. NaCl to 1 per cent, 0.6875 per cent phenol, and 1 per cent ether were added to the resulting protein fraction and the reaction was adjusted to pH 7.1. The total protein content was 16.8 Gm. per 100 ml.

Notes: a. Because it was felt that any form of filtration for the purpose of sterilization might shear off some valuable factor from the colloidal preparations, the fractions of protein described were not subjected to Berkefeld or Seitz filtration. They were refrigerated for a week and were found to be free of bacteria. This result was obtained by treating the concentration procedures with sterile precautions.

b. It was not attempted to adjust each colloidal fraction to a common total protein content. To do so would have limited the protein concentration to that of euglobulin, which, of necessity, is low. Rather, each fraction was adjusted to a maximum protein content that left it sufficiently fluid at room temperature to allow easy pipetting.

c. The calf plasma used to prepare the protein fractions described was from a single pool.

II. *Methods.*—To insure uniformity of initial viral potency in all the experiments, the virus source used was pulp from one vaccinated calf.‖

Each preparation was made by grinding 5 Gm. of pulp through a No. 60 mesh stainless steel sieve and then regrinding it through a No. 100 mesh sieve. It was then emulsified in 20 ml. of the fraction of protein to be tested. The resulting 25 ml. of virus pulp contained final concentrations of 0.5 per cent phenol and 0.8 per cent ether. One milliliter of the emulsion was dispensed into each of specially prepared dehydration ampules,[1] which were then stored at minus 28° C. overnight in a freezer. The following morning they were attached to the lyophile apparatus[18] and kept frozen throughout until the vacuum in the closed system had attained a negative pressure of 5 μ of mercury or less.

Dehydration was allowed to proceed for six hours longer at room temperature and the ampules were then sealed in vacuo. At sealing, the vacuum in our apparatus was invariably below 2 μ of mercury. The ampules were then stored at the temperatures desired for the tests.

At the end of the required period of storage an ampule was opened and the material reconstituted to original volume.[1] The dilutions indicated by the tables were made in

*The fibrin collected was used to prepare fibrin fraction 1.

†The clear filtrate was used to prepare the pseudoglobulin fraction 5.

‡Because of the relative insolubility of this type of plasma protein, it was not possible to finish with a higher protein content.

§The clear filtrate was used for the albumin preparation 6.

‖The viral potency of the pulp gave on the skin of the rabbit[17] a 20 per cent "take" at an end point dilution of 10^{-6} and 100 per cent "takes" at the lower dilutions shown in the tables.

cold saline, and the tests were performed on the depilated skin of the rabbit according to the method of Force and Leake,[17] and with the added precautions described before.[1]

RESULTS AND DISCUSSION

In a previous publication,[1] data were submitted establishing the durability to various temperatures up to 37.5° C. of a smallpox vaccine prepared in calf globulin and dehydrated. The calf globulin had been subjected to heating of 28° C. for half an hour, which was thought would inactivate, to some extent, antibodies that might be deleterious to the smallpox virus. The smallpox vaccine so prepared was subjected to a temperature of 37.5° C. for 43 days. At the end of this time its viral potency was sufficient to fulfill N.I.H. standards of infectivity[19] and was, therefore, still suitable for human vaccination.

In efforts to further improve the durability of this type of smallpox vaccine and to attempt to determine the mechanism of viral protection afforded by calf globulin, preparations were made with each of the various protein bands of calf plasma that are chemically separable. These included euglobulin, pseudoglobulin, fibrin, albumin, and two globulin fractions, one of which had been heat inactivated at 57° C. for half an hour. The vaccines were subjected to storage temperatures of 37.5° C. and 45° C., and the viral potency of each was determined on the skin of the rabbit at periodic intervals of storage time.

Comparative results of viral potency demonstrate that the vaccine prepared with unheated globulin was protected best against high temperatures (Tables I and II). After exposures of 90 days to 37.5° C. and 60 days to 45° C., it was still suitable for human vaccination, according to N.I.H. standards of infectivity[19] (Table I).

The dehydrated vaccine prepared with the globulin heat inactivated at 57° C. for one-half hour demonstrated a much lower resistance to high temperatures. Its durability was approximately the same as that demonstrated by the vaccine prepared in the globulin fraction heat-inactivated at 28° C. for one-half hour.[1]

It seems, therefore, that unheated calf globulin is the colloid of choice for preparing dehydrated smallpox vaccine. It seems evident, also, that the factor or complex present in calf globulin which affords dehydrated smallpox virus protection against high temperatures is thermolabile at a temperature as low as 28° C. when it is exposed to it for one-half hour.

To determine in which chemically separable fraction of calf globulin the thermolabile element is present, vaccines were made in the euglobulin and pseudoglobulin fractions* and tested as the others.

Each demonstrated low protective value for dehydrated smallpox virus when exposed to high temperatures (Table II). When the two protein fractions were mixed in equal portions to give a semblance of whole globulin

*Euglobulin and pseudoglobulin are the two major components of globulin.

again and a vaccine was prepared with it and tested, the protection to high temperatures afforded the virus was not improved over that afforded by the pseudoglobulin (Table II).

TABLE I. COMPARATIVE DATA DEMONSTRATING THE PROTECTIVE QUALITIES TO HIGH TEMPERATURES THAT CALF FIBRIN AND CALF GLOBULIN HAVE FOR THE SMALLPOX VIRUS

PROTEIN FRACTION USED TO MAKE THE 20% EMULSION OF PULP	PERIOD OF STORAGE		INFECTIVITY FOR THE RABBIT								
			DILUTIONS OF VACCINE MADE IN SALINE AND % "TAKES"†								
	NO. OF DAYS	TEMP. ° C.	1/10	1/100	1/M	1/3M	1/10M	1/20M	1/30M	1/40M	1/80M†
Fibrin	30	37.5*	100	100	90	80	40	40	30	20	5
		45 *	100	100	90	75	30	20	15	5	0
	45	37.5*	100	100	85	70	30	25	20	15	5
		45	100	100	55	40	20	15	10	5	0
	60	37.5*	100	100	80	60	30	10	10	10	5
		45	100	100	25	25	20	10	5	0	0
	90	37.5	100	100	55	40	20	10	5	5	–
		45	100	100	25	20	15	0	0	0	–
	120	37.5	100	100	40	30	15	5	5	5	–
		45	100	95	15	15	10	0	0	0	–
	150	37.5	100	90	25	20	5	0	0	0	–
		45	100	80	5	5	0	0	0	0	–
	210	37.5	100	45	15	10	5	0	0	0	–
		45	90	30	5	5	0	0	0	0	–
Globulin not heated	30	37.5*		100	100	100	80	35	30	15	5
		45 *		100	95	95	30	10	10	5	5
	45	37.5*		100	100	90	35	15	10	10	5
		45 *		100	95	85	20	10	10	5	5
	60	37.5*	100	100	90	80	20	10	5	0	–
		45 *	100	100	80	75	15	10	5	0	–
	90	37.5*	100	100	80	70	15	10	5	0	–
		45	100	90	30	25	5	0	0	0	–
	120	37.5	100	95	45	40	15	5	0	0	–
		45	100	65	15	10	5	0	0	0	–
	150	37.5	100	95	40	30	15	5	0	0	–
		45	100	65	15	10	5	0	0	0	–
	210	37.5	100	90	30	20	5	5	0	0	–
		45	100	60	5	5	0	0	0	0	–
Globulin heated to 57° C. ½ hr.	30	37.5*		100	100	85	60	15	10	5	0
		45 *		100	80	45	30	10	5	0	0
	45	37.5*	100	100	100	80	20	15	10	5	0
		45	100	100	40	30	10	5	0	0	0
	60	37.5	100	100	70	50	15	5	0	0	–
		45	100	95	30	25	10	5	0	0	–
	90	37.5	100	100	50	40	10	5	0	0	–
		45	100	80	20	15	5	0	0	0	–
	120	37.5	100	100	50	35	5	0	0	0	–
		45	100	40	15	10	0	0	0	0	–
	150	37.5	100	90	20	15	5	0	0	0	–
		45	100	40	15	5	0	0	0	0	–
	210	37.5	10	5	0	0	0	0	0	0	–
		45	0	0	0	0	0	0	0	0	–

*The vaccine after the period of storage at the temperature indicated still complies with N.I.H. standards of infectivity[19] and, therefore, is still suitable for human vaccination.
†M = 1,000.

Evidently, the viral protective element present in calf globulin had been lost, was denatured, or had been made dialyzable when the euglobulin and pseudoglobulin fractions were prepared with the chemical procedure described.

TABLE II. COMPARATIVE DATA DEMONSTRATING THE PROTECTIVE QUALITIES TO HIGH TEMPERATURES THAT DIFFERENT FRACTIONS OF CALF PLASMA HAVE FOR THE SMALLPOX VIRUS

PROTEIN FRACTION USED TO MAKE THE 20% EMULSION OF PULP	PERIOD OF STORAGE		INFECTIVITY FOR THE RABBIT								
			DILUTIONS OF VACCINE MADE IN SALINE AND % "TAKES"[1]								
	NO. OF DAYS	TEMP. ° C.	1/10	1/100	1/M	1/3M	1/10M	1/20M	1/30M	1/40M	1/80M†
Euglobulin	30	37.5*	100	100	90	80	15	5	5	0	0
		45	100	100	40	15	5	0	0	0	0
	45	37.5	100	100	40	30	15	5	0	0	0
		45	100	100	30	20	5	0	0	0	0
	60	37.5	100	90	40	20	0	0	0	0	0
		45	90	15	0	0	0	0	0	0	0
	90	37.5	100	85	35	10	0	0	0	0	0
		45	80	10	0	0	0	0	0	0	0
	120	37.5	100	70	25	5	0	0	0	0	0
		45	60	35	0	0	0	0	0	0	0
	150	37.5	100	60	25	0	0	0	0	0	0
		45	60	60	0	0	0	0	0	0	0
	210	37.5	95	60	10	0	0	0	0	0	0
		45	0	0	0	0	0	0	0	0	0
Pseudoglobulin	30	37.5*	100	100	100	95	20	5	5	5	5
		45 *	100	100	90	80	15	5	5	0	0
	45	37.5	100	100	95	90	5	0	0	0	0
		45	100	70	50	45	5	0	0	0	0
	60	37.5	100	90	40	30	5	0	0	0	0
		45	100	60	15	10	5	0	0	0	0
	90	37.5	100	85	25	20	5	0	0	0	0
		45	100	40	15	5	5	0	0	0	0
	120	37.5	100	85	25	20	5	0	0	0	0
		45	100	35	10	0	0	0	0	0	0
	150	37.5	100	85	10	5	5	0	0	0	0
		45	100	35	10	0	0	0	0	0	0
	210	37.5	95	80	10	0	0	0	0	0	0
		45	75	10	5	0	0	0	0	0	0
Pseudoglobulin and euglobulin mixed, 1-1	30	37.5*	100	100	100	90	60	10	5	5	–
		45 *	100	100	75	80	20	10	5	5	–
	45	37.5	100	100	60	50	40	5	0	0	–
		45	100	95	20	20	15	5	0	0	–
	60	37.5	100	100	55	50	20	5	0	0	–
		45	100	70	20	15	5	0	0	0	–
	90	37.5	100	100	40	30	5	0	0	0	–
		45	95	30	15	10	0	0	0	0	–
	120	37.5	100	100	35	30	5	0	0	0	–
		45	90	30	10	5	0	0	0	0	–
	150	37.5	100	90	20	15	5	0	0	0	–
		45	70	30	10	5	0	0	0	0	–
	210	37.5	90	15	0	0	0	0	0	0	–
		45	20	10	0	0	0	0	0	0	–
Albumin	30	37.5*	100	100	100	90	50	40	30	15	–
		45 *	100	100	85	80	15	15	10	10	–
	45	37.5	100	100	45	40	20	5	5	0	–
		45	100	95	35	30	0	0	0	0	–
	60	37.5	100	100	45	40	10	5	0	0	–
		45	100	85	35	30	0	0	0	0	–
	90	37.5	100	100	40	30	5	0	0	0	–
		45	100	70	35	15	0	0	0	0	–
	120	37.5	100	65	20	10	5	0	0	0	–
		45	90	40	15	10	0	0	0	0	–
	150	37.5	100	40	15	5	5	0	0	0	–
		45	90	30	10	5	0	0	0	0	–
	210	37.5	100	35	10	5	5	0	0	0	–
		45	85	30	5	0	0	0	0	0	–

*The vaccine after the period of storage at the temperature indicated still complies with N.I.H. standards of infectivity[19] and, therefore, is still suitable for human vaccination.
†M = 1,000.

The dehydrated vaccine prepared with the albumin fraction of calf plasma gave approximately the same low durability against high temperatures as the one prepared with the pseudoglobulin fraction (Table II).

The dehydrated vaccine prepared with the fibrin fraction of calf plasma demonstrated a surprising longevity when it was exposed to high temperatures, especially to 37.5° C. (Table I). The durability was higher than that demonstrated by the vaccines prepared with the other protein bands of calf plasma (Tables I and II), with the exception of the one prepared with the calf globulin that had not been heat inactivated (Table I).

The bacterial sterility of all the vaccines prepared for the experiments conformed to the standards of the N.I.H.,* even though the calf pulp used for the preparations had a bacterial content of "uncountable." It is stressed again,[1] however, that the antiseptic Roccal was sprayed daily on the abdomen of the vaccinated calves during the viral incubation period. The amount of this reagent that may be adsorbed by the pulp and that may be an additive to the bactericidal effect of the phenol and ether used in the preparation of the vaccines is still being investigated in our laboratory.

SUMMARY

A dehydrated smallpox vaccine prepared with calf globulin is described. The vaccine had sufficient viral potency to warrant its use for human vaccination after exposures of 90 days to 37.5° C. and 60 days to 45° C.

Dehydrated smallpox vaccines prepared with the euglobulin, pseudoglobulin, and albumin fractions of plasma demonstrated relatively low resistance to high temperatures.

A dehydrated smallpox vaccine prepared with the fibrin fraction of calf plasma demonstrated good resistance to high temperatures.

Data submitted suggest that the element contained in calf globulin that has protective qualities for dehydrated smallpox virus when it is exposed to high temperatures is thermolabile.

Simultaneously with the preparation of the vaccines, the bacterial count of each was diminished to an acceptable figure in spite of the use of highly contaminated calf pulp as the virus source.

The author acknowledges the assistance rendered by Magda Gagliardi and Marie Mazzochi in the performance of the many animal tests and other technical work.

REFERENCES

1. Cardone, E.: The Stability of the Viral Potency of Smallpox Vaccine When Prepared in Calf Globulin, Dehydrated, and Stored Without Refrigeration, J. LAB. & CLIN. MED. **42**: 693-699, 1953.
2. Green, A. B.: The Influence of Temperature and Some Other Physical Conditions on Calf Vaccine, J. Hyg. **8**: 525-535, 1908.
3. Hornibrook, J. W., and Gerhard, W. H.: Dried Smallpox Vaccine, Pub. Health Rep. **66**: 38-43, 1951.
4. Hornibrook, J. W.: A Useful Menstruum for Drying Organisms and Viruses, J. LAB. & CLIN. MED. **35**: 788-792, 1950.

*The National Institute of Health standards require a smallpox vaccine used for human vaccination to have a bacterial count of 1,000 per milliliter or less.

5. Goodpasture, E. W., and Buddingh, G. J.: The Preparation of Antismallpox Vaccine by Culture of the Virus in the Chorio-Allantoic Membrane of Chick Embryos and Its Use in Human Immunization, Am. J. Hyg. **21**: 319-360, 1935.
6. Goodpasture, E. W., and Buddingh, G. J.: The Protective Action of Rabbit Serum for Vaccinia Virus at High Temperatures, Science **84**: 66-67, 1936.
7. Buddingh, G. J.: Production and Use of Smallpox Vaccine Virus Cultivated in the Chorio-Allantoic Membrane of Chick Embryos, Am. J. Pub. Health **27**: 1135-1140, 1937.
8. Buddingh, G. J.: The Use of Serum as a Protective Diluent for Vaccinia Virus, Am. J. Hyg. **27**: 530-537, 1938.
9. Lloyd, W., and Mahaffy, A. F.: Cultivation of Vaccinia Virus by River's Method, Proc. Soc. Exper. Biol. & Med. **33**: 154-156, 1935.
10. Rivers, T., and Ward, S. M.: Jennerian Prophylaxis by Means of Intradermal Injections of Culture Vaccine Virus, J. Exper. Med. **62**: 549-560, 1935.
11. Morozov, M. A., Korolkova, M. I., Kasatkevich, S. S., and Dolinov, K. E.: Dry Smallpox Vaccine, Zhurnal Mikrobiol. Epidmiol. i. Immunobiol. **6**: 76-80, 1943.
12. Nagano, Y.: Sur Une Proprieté Particuliere De La Lymphe Vaccinale Desséchée, Jap. J. Exper. Med. **20**: 37-42, 1949.
13. Otten, L.: Trockenlymphe, Ztschr. Hyg. **107**: 677-696, 1927.
14. Otten, L.: Trockenlymphe, Ztschr. Hyg. **114**: 705-719, 1933.
15. Collier, L. H.: The Development of a Stable Smallpox Vaccine, J. Hyg. **53**: 76-101, 1955.
16. Collier, W. A.: Vaccine Antivarioliques Secs; Boletin Ofic. San Panam. **34**: 143-163, 1953.
17. Force, J. N., and Leake, J. P.: A Method for Estimating the Potency of Smallpox Vaccine, Hyg. Lab. Bull. U. S. Pub. Health Service **149**: 1-27, 1927.
18. Flosdorf, E. W., and Mudd, S.: Procedure and Apparatus for Preservation in "Lyophile" Form of Serum and Other Biological Substances, J. Immunol. **29**: 389-425, 1935.
19. Federal Security Agency, National Institute of Health, Bethesda, Maryland, Jan. 1, 1951, Minimum Requirements: Smallpox Vaccine, 3rd Revision.

LABORATORY METHODS

A SIMPLE SCREENING TEST FOR ELIMINATION OF ARTIFACTUALLY HIGH VALUED PROTEIN-BOUND IODINE SAMPLES

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ONE of the difficulties in performing large numbers of protein-bound iodine determinations is the danger of contamination of the laboratory or individual apparatus with extraneous iodine. Precautions have been emphasized by various authors, but in spite of the utmost care in technique, contamination leading to artifactually high PBI values occasionally occurs. In our experience, the cause is usually contamination from one or more specimens of blood from patients who have received radiopaque organic iodine for roentgenographic procedures. These organic iodides are in the form of Diodrast, Urodrast, Lipiodol, and other similar commercial products. Following their administration we have found them to be present in the serum of such patients from ten days to three years, depending upon the type of contrast material administered and its locus of concentration, although there appears to be no simple rule of thumb for predicting the time before the serum is completely cleared of these substances or their derivatives. We think it is likely that it is taken up by the reticuloendothelial system and released at varying rates in different people.

The dry ash method[1] of analyzing protein-bound iodine in serum appears to be particularly prone to having several determinations in a single lot of samples rendered invalid because of cross contamination from a specimen containing this type of organic iodine in some form. We agree with Samson and associates[2] that most of the difficulty occurs in the muffle furnace during the ashing procedure.

In order to eliminate the possibility of such contamination we have devised a semiquantitative screening process which will detect the "guilty" specimens. The method uses only three drops of serum and can be completed in about forty minutes. The process depends upon partial hydrolysis of the serum proteins in 8 N KOH with the breaking of peptide bonds and the liberation of some of the artifactual iodine. (The fact that this iodine cannot be washed free from the proteins by physical means makes it likely that it is attached by some type of chemical bond rather than being simply bound by

Received for publication June 11, 1955.
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a process of adsorption.) After this process we use the standard reaction common to all PBI analyses, utilizing the catalytic action of iodine, probably as iodide on the reduction of yellow ceric ion to colorless cerous ion by arsenious acid in the presence of hydrogen ion.

MATERIALS AND METHOD

Materials.—

1. *8 N KOH:* Dissolve 527 Gm. of KOH pellets (85 per cent) in water and dilute to 1 liter.
2. *Ceric ammonium sulfate (0.08 N) (G. Frederick Smith Co.):* Dissolve 50.62 Gm. of ceric ammonium sulfate [$(NH_4)_4Ce(SO_4)_4 2H_2O$] and 100 ml. of concentrated sulfuric acid in 400 ml. of water. Dilute to 1 liter.
3. *Arsenious acid (0.2 N):* Dissolve 9.9 Gm. of arsenious trioxide (As_2O_3) and 2.4 Gm. NaOH (pellets) in 100 ml. of H_2O by boiling. Dilute to approximately 700 ml., add 8.5 ml. of 7 N H_2SO_4, and dilute to 1 liter.

Method.—

1. Add 2 drops of 8 N KOH to 3 drops of each sample of serum in a 13 × 100 mm. test tube placed in a Kahn rack.
2. Place the rack in a boiling water bath for 30 minutes. The tubes are covered with small glass marbles to minimize evaporation of liquid.
3. After 30 minutes the tubes are removed, and each tube has added to it 0.5 ml. of distilled H_2O, followed by 1.0 ml. of 0.2 N arsenious acid and then 1.0 ml. of 0.08 N ceric ammonium sulfate.
4. Mix each solution well at room temperature and wait five minutes.
5. Read against white background.

RESULTS

Those specimens which contain PBI values of over 800 meg. per cent and which can be assumed to be contaminated with artifactual iodide will decolorize to such an extent in this period of time that the differentiation between these specimens and normal and pathologically high samples is quite simple. We recommend that a contaminated specimen be used as a positive control in each screening series.

DISCUSSION

Although the protein is not sufficiently hydrolyzed during this process to prevent reprecipitation when the acid ceric sulfate is added, the precipitate does not interfere appreciably with the final reading of this screening test. There have been occasions where artifactually high PBI values in the order of approximately 100 meg. per cent have not been detected, but these occurrences are rare since most contaminated specimens have been in the order of 800 to 5,000 meg. per cent. In any case, even though these low order contaminations are not detected, they have in no case raised the level of values of the other specimens. We feel that it is necessary that the sensitivity of the test be low enough so that high values due to hyperthyroidism (8 to 35 meg. per cent) are not falsely interpreted as being artifactual.

This screening procedure offers several advantages over the Samson procedure, the most important being that the highly contaminated specimens are detected before the quantitative assay is started and thus prevents any possible cross contamination during any of the steps preceding the ashing process. Other advantages are the elimination of the possibility of contamination of the furnace and the shorter time involved in the entire screening procedure.

SUMMARY

A simple semiquantitative analysis is presented for detecting artifactually high serum protein-bound iodine due to iodine containing contrast media used in roentgenographic procedures. This method has a minimum number of steps, requires no elaborate apparatus, and can be completed in approximately forty minutes. Because it allows initial detection of contaminated specimens it is used to screen out such samples and thus prevent undesirable cross contamination before the quantitative assay is started.

I wish to thank Elaine Freeberg Swartz for technical assistance in these experiments.

REFERENCES

1. Barker, S. B., Humphrey, M. J., and Soley, N. H.: The Clinical Determination of Protein-Bound Iodine, J. Clin. Invest. **30**: 55-62, 1951.
2. Samson, M., Brown, H., and Eichen, S.: Cross-Contaminator Exclusion Test for Dry-Ashing Determination of Protein-Bound Iodine, Clinical Chemistry **1**: 269-272, 1955.

FLAME PHOTOMETRIC DETERMINATION OF SERUM CALCIUM

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INTRODUCTION

VARIOUS methods have been proposed for the determination of serum calcium. The classical method of Kramer and Tisdall[1] and its various modifications give accurate and precise results. However, like all methods depending upon oxalate precipitation of the calcium, this method is time consuming, and generally several hours must elapse before laboratory results become available to the clinical staff of the hospital.

In recent years new methods for the determination of serum calcium have been reported involving colorimetric,[2, 3] complexiometric,[4-7] or flame photometric[8-13] procedures. These numerous recent publications dealing with the determination of calcium in biologic fluids indicate an urgent need for a convenient, quick, and precise method. The present procedure was developed after many of the above methods had been tried in this laboratory and found to be inadequate for our purpose.

EXPERIMENTAL

Reagents and Equipment.—The highest grade of reagents obtainable was used. All solutions were made up in double distilled water and, with the exception of those to be used immediately, were kept in polyethylene bottles. A Beckman spectrophotometer Model DU, equipped with acetylene-oxygen flame attachment and photomultiplier tube, was used for all determinations.

Trichloroacetic Acid 6.2 Per Cent.—Dissolve 31 Gm. of trichloroacetic acid (TCA) in water and dilute to 500 ml.

Organic Diluting Fluid.—To 500 ml. of isopropyl alcohol (99 per cent) add 300 ml. of distilled water and 0.2 ml. of Sterox SE.* Mix well. We have not found it necessary to store this solution in polyethylene containers.

Mixed Stock Standard (MSS).—This solution contains 150 milliequivalents (meq.) of sodium, 5 meq. potassium, and 5 meq. calcium per liter. It is prepared by dissolving 0.2503 Gm. of Iceland Spar in 25 ml. of 2 N HCl in a liter volumetric flask and diluting to approximately 500 ml. with water. After 8.7690 Gm. of sodium chloride and 0.3728 Gm. of KCl are dissolved in this solution, it is diluted to volume with water. When the solution was stored in tightly stoppered polyethylene bottles, no changes in composition could be detected, even after months of storage.

Immediately before flame photometric determinations are to be carried out, the following working standards are prepared from the mixed stock standard. For Standard 1, 1.00 ml. of MSS and 9.0 ml. TCA are added to a glass-stoppered 50 ml. volumetric flask and diluted to

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Received for publication Nov. 5, 1955.

*Monsanto Chemical Company, St. Louis, Mo.

volume with the organic diluting fluid. For Standard 2, 0.80 ml. of MSS, 0.2 ml. of water, and 9.0 ml. of TCA are diluted to 50.0 ml. with the organic diluting fluid. For Standard 3, 0.60 ml. of MSS, 0.4 ml. of water, and 9.0 ml. of TCA are diluted to 50.0 ml. with the organic diluting fluid. The blank is prepared by substituting 1.0 ml. of water for the MSS in Standard 1. With the instrument used in the present study the following settings were found to be satisfactory: wave length dial 422.7 mμ; slit width 0.036 mm.; selector switch 0.1 position; photomultiplier sensitivity, full position; resistor selector 22 megohms; oxygen 9 pounds; and acetylene 7 pounds.

PROCEDURE

The proteins are precipitated by adding 0.5 ml. of serum to 4.5 ml. of the TCA solution contained in conical centrifuge tubes. After thorough mixing of the contents, the tubes are centrifuged for 10 minutes, and 2.0 ml. aliquots of the supernatant are then added to calibrated glass-stoppered cylinders and diluted to 10.0 ml. with the organic diluting fluid. The samples are kept in these stoppered cylinders until just prior to evaluating them photometrically.

In the meantime the spectrophotometer has been turned on and allowed to warm up. The blank and the standards are prepared as indicated. The final adjustments of the instrument are made so that the blank solution will read 0 luminosity units (LU) and Standard 1 will give a reading of 50 LU (using the percentage transmittancy scale of the instrument). Standards 2 and 3 are then introduced and should give readings of 40 and 30 LU ± 1, respectively (Fig. 2).

After the instrument has been calibrated thus, the luminosity of the unknown sample is measured. From the luminosity values thus obtained, the amount of calcium in the original serum samples is calculated by use of the relationship: LU/10 = milliequivalents of calcium per liter of serum. As a precautionary measure against possible instrument drift, the readings obtained with the blank and Standard 1 are verified after every three or four serum samples have been measured. One of the advantages of the proposed method is that a sample does not have to be discarded after each reading but may be used over again several times without affecting its luminosity reading.

DISCUSSION OF RESULTS

The proposed method is a modification of that described by Kingsley and Schaffert,[10, 14] who first used Sterox and organic diluting fluids in flame photometry. Sterox presumably serves to keep the capillary of the atomizer clean, while the organic diluting fluid increases the luminosity of calcium by the combined effect of increasing the temperature of the flame and by decreasing the viscosity of the solution. Kingsley and Schaffert advocate the use of a diluting fluid containing acetone, acetic acid, and aqueous Sterox, which they found to be very effective in increasing the luminosity of the flame. This observation was verified, yet the Kingsley-Schaffert diluting fluid was found to be unsuitable for routine use in this laboratory for several reasons. Thus the rapid rate of evaporation of acetone causes changes in the concentration of the unknown solution, if the solution in the beaker of the flame photometer is allowed to stand uncovered or loosely covered for more than a few minutes at room temperature. Furthermore, it was found that when the sample was introduced under the atomizer, the rapid evaporation and/or aspiration of the sample made it impossible to obtain checks on the luminosity readings thirty seconds apart. Another disadvantage of this diluting fluid is that when the sample is removed from underneath the atomizer the flame

very frequently blows out. Winer and Kuhns,[11] who used the Kingsley-Schaffert diluting fluid, also noted these frequent blowouts and sought to overcome them by squirting water into the beaker prior to its removal from the atomizer. If the sample is diluted with sufficient water the blowouts can be prevented, but in our hands the procedure was inconvenient, particularly so when many samples had to be run, and obviously a sample is ruined by this procedure so that its luminosity cannot be checked or rechecked.

In the Kingsley-Schaffert method the serum is added directly to the organic diluting fluid. The findings of Kingsley and Schaffert that the presence of proteins in the final solution does not affect the luminosity of calcium were confirmed. It was also found, however, that while discrete precipitation of proteins occurred only very rarely, faint turbidity was produced much more frequently. Such samples when atomized had a tendency to foul the capillary of the burner, causing erratic readings. Altering the Sterox concentration failed to prevent this annoyance and only an exhaustive cleaning process could put the burner in service again.

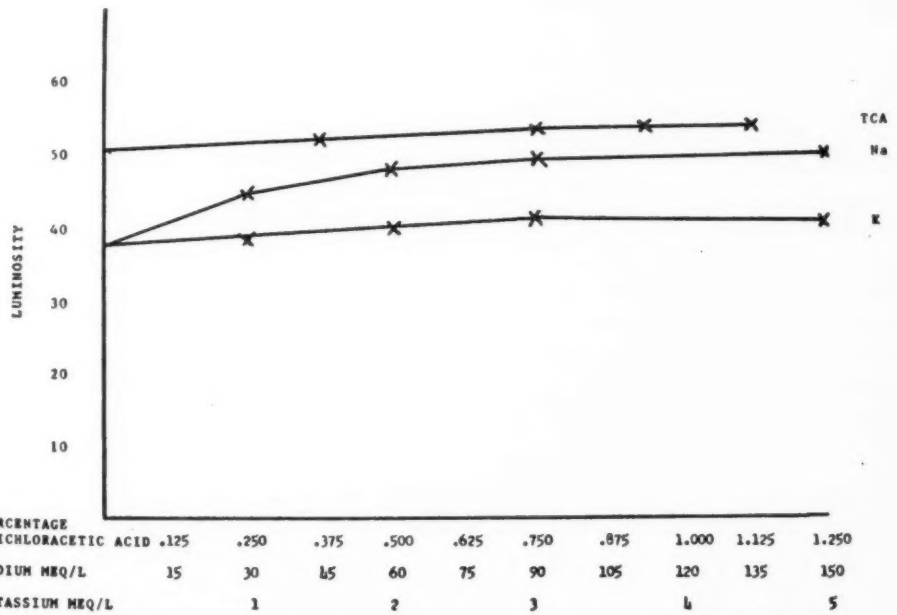

Fig. 1.—Factors influencing the luminosity of calcium in the proposed method. Before dilution of 1:50 with the organic diluting fluid, all samples contained 5.0 meq./liter calcium, while the TCA, sodium, and potassium concentrations were varied, as indicated in the figure.

Precipitation of Protein.—It was decided to avoid the disturbing effect of proteins by precipitating them prior to the addition of the organic dilution fluid. In the method as finally adopted, the proteins are precipitated by TCA. The filtrate obtained neither causes a turbidity when diluted with the organic solvent nor does it contaminate the burner capillary even after hundreds of samples have been analyzed. Although it was found that in the concentration

used, TCA does not affect the luminosity of calcium significantly for routine use (Fig. 1), equivalent amounts of TCA are added to the standards as a precaution to cancel out the effect of possible trace contamination.

Selection of Organic Diluting Fluid.—Acetone intensifies the luminosity of calcium particularly when it is used in 80 to 90 per cent concentration. Isopropyl alcohol in similar concentration is less effective in this respect,[10] but the difference in effect between the two is much less when lower concentrations (50 per cent) of these two solvents are compared. Considering the relatively high vapor pressure of acetone, it is rather difficult to prevent evaporation losses in the summertime under conditions of routine analysis. Isopropyl alcohol with its higher boiling point would seem to be less subject to vaporization errors than acetone, yet care must be taken not to expose solutions of it to the air for any length of time. An experiment, the results of which are listed in Table I, emphasizes this point. Filtrates prepared with the organic

TABLE I. EVAPORATION OF ORGANIC DILUTING FLUID IN 30 MINUTES AT ROOM TEMPERATURE (30° C.)

CONTAINER*	VOLUME (ML.)	PERCENTAGE LOSS‡
Beaker, uncovered	3.0	3.9
Beaker, covered	3.0	0.8
Test tube, uncovered	10.0	0.006
Beaker, uncovered, siliconed†	3.0	3.6
Beaker, covered, siliconed†	3.0	0.9

*Standard 5 ml. beakers used for the flame photometer.
†Desicote, a Beckman Instrument Co. preparation, was used.
‡Average of three determinations.

diluting fluid according to the proposed procedure were placed in standard 5 ml. beakers or in test tubes and kept at room temperature (30° C.) for thirty minutes. The loss of weight was then determined. It was noticed early in this study that the organic fluids, particularly those containing acetic acid, have a tendency to "creep" up on the sides of the beakers, irrespective of their being coated with silicone. Thus not only is a larger surface of solution exposed to evaporation, but actually some of the liquid may reach the cover glass and even the outside of the beaker. In the method as finally adopted, an isopropyl alcohol concentration of 50 per cent was chosen because it was found that higher concentrations, while increasing the luminosity, caused flame blowouts; lower concentrations on the other hand reduced the luminosity of the calcium to an undesirable level.

Interference of Sodium and Potassium.—Sodium and potassium definitely affect the luminosity of calcium in flame photometry. But this effect is only significant when the luminosity of calcium in the presence of these ions is compared with that obtained in their complete absence. It will be noted in Fig. 1 that changes in sodium and potassium concentration within the physiologic range have only a slight effect on the luminosity of calcium and for routine studies can be ignored if the standards are prepared as specified.

Recovery Studies.—The results shown in Fig. 2 demonstrate that the proposed method gives a linear response with increasing calcium concentrations. Hence the use of the simple formula LU/10 = milliequivalents of calcium per liter of serum is justified. In this figure the two sets of data are shown. The results represented by open circles were obtained in the usual manner; that is, the technician running the test knew that she was determining a luminosity-concentration curve. The other values plotted represent unknowns which were submitted to the same technician, who was not aware that when completed, the results should show a straight-line luminosity-concentration relationship. The slight deviation shown in the figure demonstrates the experimental error which may be expected when the procedure is carried out by a qualified and experienced technician. In Table II are shown a part of the results obtained in the recovery studies. In these studies some of the samples were prepared from plasma obtained from out-dated human blood and fortified with various amounts of calcium. In other cases serum samples were analyzed by the proposed method as well as by the Kramer and Tisdall method[1] and the difference in results expressed as percentage error in the appropriate column. It will be noted that the accuracy of the proposed method is quite adequate for all clinical purposes.

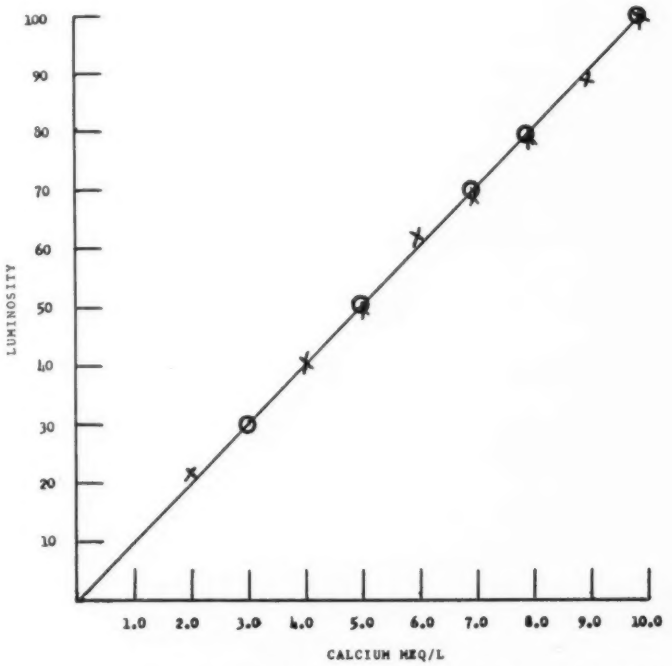

Fig. 2.—Concentration-luminosity relationship in the proposed method.

The method as described has been used mainly in clinical studies. It requires 0.5 ml. of serum for duplicate determinations. Adequate luminosity readings can be obtained with the Beckman flame photometer with as little as 2 ml. of final solution. Since the final volume in the proposed method is 10

ml., it is obvious that it can readily be adapted to the use of smaller quantities of serum if desirable and if the necessary calibrated glassware is available.

TABLE II. RECOVERY STUDY BY THE PROPOSED METHOD

MEQ./L. Ca^{++} CALCULATED	MEQ./L. Ca^{++} FOUND	PERCENTAGE ERROR
4.6	4.6	0.0
6.8	7.0	+2.9
6.2	6.1	−1.6
5.1	5.2	+2.0
5.7	5.8	+1.8
6.2	6.1	−1.6
5.6	5.7	+1.8
6.7	6.5	−3.0
4.5*	4.5	0.0
4.8*	4.7	−2.1
4.3*	4.2	−2.3
4.5*	4.8	+6.7
4.6*	4.5	−2.2
4.2*	4.1	−2.4
4.7*	4.6	−2.1
4.4*	4.6	+4.5

*Serum samples. Values by the Kramer-Tisdall method.

SUMMARY

A convenient and quick method for the determination of serum calcium is proposed. It involves precipitation of the proteins with TCA and dilution of aliquots of the filtrate with isopropyl alcohol to a final concentration of 50 per cent. It avoids some of the undesirable features of previous methods employing organic solvents, such as flame blowouts, contamination of atomizer by proteins, and rapid evaporation of the solvents. The precision and accuracy of the proposed method are adequate and it is readily adaptable to serum samples smaller than 0.5 ml.

REFERENCES

1. Kramer, B., and Tisdall, F. F.: J. Biol. Chem. **48:** 223, 1921, as cited by Koch, F. C., Practical Methods in Biochemistry, ed. 3, Baltimore, 1941, Williams & Wilkins Company, p. 165.
2. Natelson, S., and Penniall, R.: Colorimetric Estimation of Ultramicro Quantities of Calcium in Human Serum as the Complex With Alizarin, Anal. Chem. **27:** 434, 1955.
3. Makepeace, U.: Colorimetric Determination of Serum Calcium, J. Biol. Chem. **199:** 251, 1952.
4. Aconsky, L., and Mori, M.: Spectrophotometric Technique for Calcium, Anal. Chem. **27:** 1001, 1955.
5. Young, A., and Sweet, T. R.: Complexes of Eriochrome Black T With Calcium and Magnesium, Anal. Chem. **27:** 418, 1955.
6. Fales, F. W.: A Micromethod for the Determination of Serum Calcium, J. Biol. Chem. **204:** 577, 1953.
7. Buckley, E. S., Gibson, J. G., and Bortolotti, T. R.: Simplified Titrimetric Techniques for the Assay of Calcium and Magnesium in Plasma, J. LAB. & CLIN. MED. **38:** 751, 1951.
8. Chen, P. S., and Toribara, T. Y.: Determination of Calcium in Biological Material by Flame Photometry, Anal. Chem. **25:** 1642, 1953.
9. Chow, T. J., and Thompson, T. G.: Flame Photometric Determination of Calcium in Sea Water and Marine Organisms, Anal. Chem. **27:** 910, 1955.
10. Kingsley, G. R., and Schaffert, R. R.: Micro Flame Photometric Determination of Sodium, Potassium, and Calcium in Serum with Organic Solvents, J. Biol. Chem. **206:** 807, 1954.

11. Winer, A. D., and Kuhns, D. M.: Calcium Determination by Flame Spectrophotometry, Am. J. Clin. Path. **23**: 1259, 1953.
12. Rothe, C. F., and Sapirstein, L. A.: A Self-Standardization Method for Flame Spectrophotometric Determination of Calcium in Biologic Materials, Tech. Bull. Reg. Med. Tech. **25**: 184, 1955.
13. Severinghaus, J. W., and Ferrebee, J. W.: Calcium Determination by Flame Photometry; Methods for Serum, Urine, and Other Fluids, J. Biol. Chem. **187**: 621, 1950.
14. Kingsley, G. R., and Schaffert, R. R.: Direct Microdetermination of Sodium, Potassium, and Calcium, Anal. Chem. **25**: 1738, 1953.

SYRINGE MECHANISM FOR SIMULTANEOUS BLOOD SAMPLING AT CONSTANT RATES

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REGIONAL blood flow and volume measurements in the intact animal frequently require the integration of curves of concentrations of tracer substances introduced into the blood stream. The Kety and Schmidt[1] nitrous oxide method for the measurement of cerebral blood flow and the equilibration technique for the measurement of splanchnic blood volume[2] are examples of such methods.

In small animals, these techniques require the withdrawal of excessive amounts of blood, since their usual application requires intermittent sampling of an artery and a vein over a period of many minutes. Integration is then accomplished using plotted concentrations.

Scheinberg and Stead[3] introduced a modification of the nitrous oxide method which consisted of drawing continuous self-integrating blood samples during the measurement period. If a blood sample is drawn at a perfectly constant rate from a blood vessel, the concentration of any substance in that sample will be the mean concentration for the collection period, despite large fluctuations in concentration within that period.

This communication describes a syringe mechanism for the constant rate withdrawal of small blood samples from one or two blood vessels for use in regional blood flow or volume measurements of this type.

MATERIALS

As shown in Fig. 1, syringes are taped into V-notches in Bakelite blocks. Similar blocks are provided on the opposite side (*A*) since the apparatus may be activated in either direction. A brass tube (*B*) is inserted between opposite holding blocks. This tube is 4½ inches long, 1 inch in diameter, and contains a ⅜ inch screw (16 turns per inch) running its entire length. A 5/16 inch slot (*C*) is cut lengthwise in the brass tube, through which the adapter for attaching syringe handles (*D*) is secured to a nut riding on the long screw. The direction of travel of the nut is determined by the direction of turn of the long screw. The long screw is turned with a worm and worm-wheel (*E*) with a 40 to 1 reduction from the 833 r.p.m. shaft of a 5,000 r.p.m. variable speed reduction motor (*F*).

Adjustable microswitches (*G*) are mounted at the extreme ends of nut travel and are normally closed. These prevent damage to syringes by automatically turning off the motor when they are opened by pre-set pegs attached to the adapter (*D*).

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Supported by Research Grant H-1904 from the National Heart Institute, National Institutes of Health, Bethesda, Md.

Received for publication Dec. 12, 1955.

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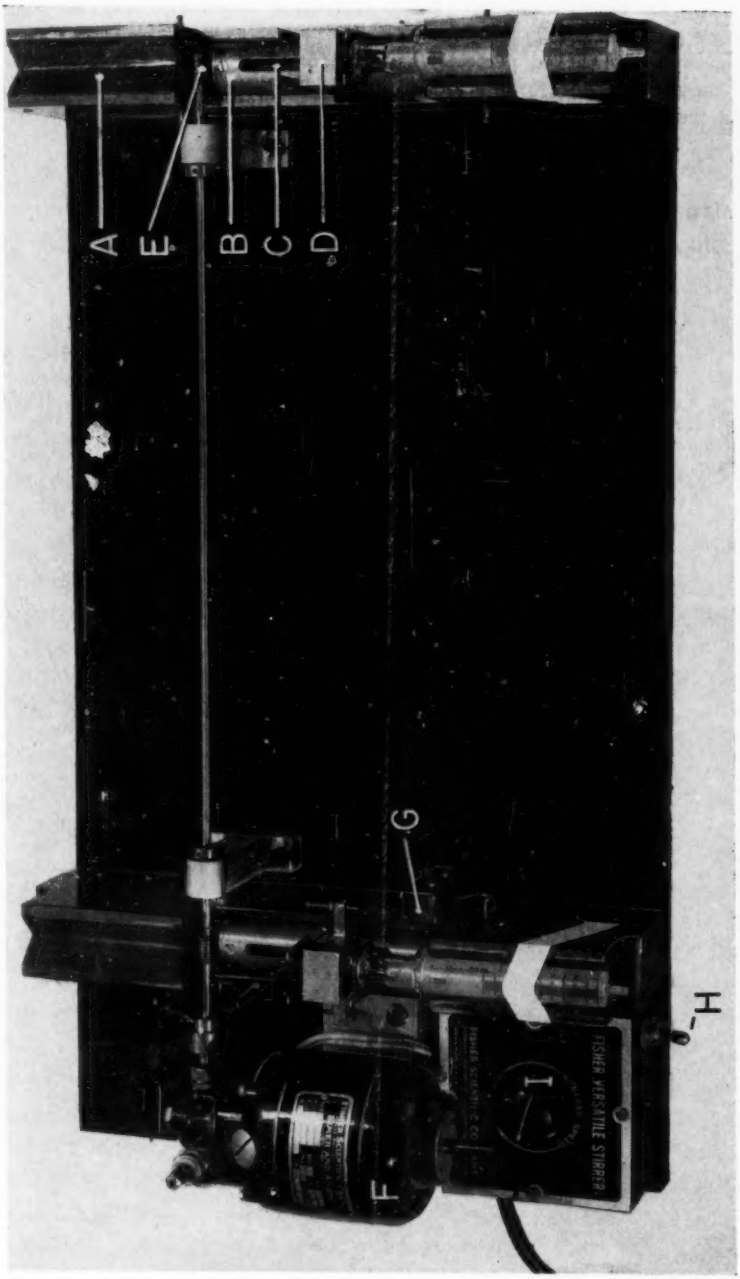

Fig. 1.—See text for discussion.

The instrument is provided with a 3-way switch (*H*) with positions for "off" and for each direction of travel, and a rheostat (*I*), which controls the speed of the motor.

With use of 10 ml. syringes, sampling at a constant rate may be accomplished between rates of 0.3 and 2.0 ml. per minute. Rates outside of this range may be obtained using smaller or larger syringes.

The apparatus has proved perfectly suitable for the types of measurements described. Details of a method for measuring cerebral blood flow in monkeys with use of indwelling catheters, and the results obtained, will be described in a later communication.

SUMMARY

An instrument is described which permits simultaneous sampling of small volumes of blood from different blood vessels at constant rates.

REFERENCES

1. Kety, S. S., and Schmidt, C. F.: The Determination of Cerebral Blood Flow in Man by the Use of Nitrous Oxide in Low Concentrations, Am. J. Physiol. **143**: 53-66, 1945.
2. Bradley, S. E., Marks, P. A., Reynell, P. C., and Meltzer, J.: The Circulating Splanchnic Blood Volume in Dog and Man, Tr. A. Am. Physicians **66**: 294-302, 1953.
3. Scheinberg, P., and Stead, E. A., Jr.: The Cerebral Blood Flow in Male Subjects as Measured by the Nitrous Oxide Technique. Normal Values for Blood Flow, Oxygen Utlization, Glucose Utilization, and Peripheral Resistance, With Observations on the Effect of Tilting and Anxiety, J. Clin. Invest. **28**: 1163-1171, 1949.

AN ENZYMATIC SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF PYRUVIC ACID IN BLOOD

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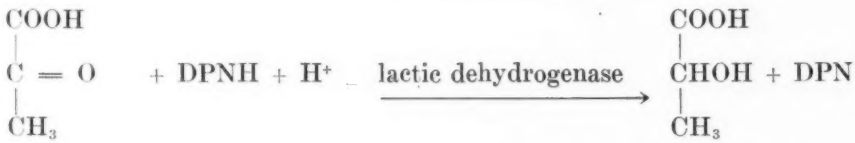
METHODS currently in use for the determination of pyruvic acid in blood are based on the reaction of this acid with dinitrophenylhydrazine. Because other keto acids present in blood also react with this reagent, additional procedures are required for separation of pyruvic acid dinitrophenylhydrazones from those of other keto acids in order to achieve specificity. To this end Friedemann and Haugen[4] have employed differential solvent extraction, while Cavallini and Frontali,[1] and Seligson and Shapiro[16] have advocated paper chromatographic separation. These additional procedures have increased the complexity of the methods and the time required for their operation.

Ochoa, Mehler, and Kornberg,[15] and Horecker and Smyrniotis[9] have utilized the enzymatic reduction of pyruvic acid to lactic acid by purified lactic dehydrogenase for the quantitative determination of pyruvic acid in other biologic systems. This reduction employs reduced diphosphopyridine nucleotide (DPNH) as hydrogen donor and proceeds according to the reaction:



The equilibrium point is far to the right.* DPNH has a strong absorption maximum at 340 mμ, and a change in optical density at this wave length is a measure of the DPNH disappearing during the reaction. Since the quantity of DPNH disappearing is stoichiometrically related to the quantity of pyruvate reduced, the magnitude of the optical density change, together with knowledge of the molar extinction coefficient for DPNH, permits calculation of the quantity of pyruvate originally present.

By an adaptation of this procedure, a simple, sensitive, and accurate method for the determination of blood pyruvic acid has been devised. Similar methods have apparently been used by other investigators.[7, 10, 17] One such, that of Hess,[7] employs the lactic dehydrogenase normally present in serum as the enzyme catalyst. The present report describes the enzymatic method used in this laboratory for the estimation of pyruvic acid in whole blood.

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.
Received for publication Dec. 19, 1955.
*The equilibrium constant is 1.7×10^{4}.[19]

METHOD

Reagents.—

1. Phosphate buffer 0.1 M, pH 7.0-7.4.

2. DPNH, prepared by hydrosulfite reduction of DPN,[11] is used as a solution of about 2 μM per milliliter. DPN was obtained from Pabst Laboratories, Milwaukee, Wisconsin. DPNH, in powdered form, can be purchased from Sigma Chemical Company, St. Louis, Missouri, and made into a solution of 2 μM per milliliter. The solution of DPNH is stored at –20° C.

3. Lactic dehydrogenase. Three different preparations were employed and all were satisfactory. The original preparation was a gift of Dr. Bernard Horecker. Later preparations were either prepared from rabbit muscle according to Kornberg[13] or purchased as a crystalline suspension from Worthington Biochemical Company, Freehold, New Jersey. Diluted enzyme is kept at –20° C.

4. Seven per cent perchloric acid.

5. Five N KOH.

Procedure.—

Blood is drawn with a minimum of stasis and transferred immediately to a small flask. Five milliliters is then rapidly withdrawn by pipette and delivered into a 15 ml. rubber-stoppered centrifuge tube containing 5 ml. of 7 per cent $HClO_4$.

The protein precipitate is removed by centrifugation and the supernatant solution is then centrifuged again. Two milliliters of clear supernatant solution is placed in a small beaker in ice. While the sample is kept cold, the pH is adjusted to 3 or 4 by the cautious addition of 5 N KOH. About 0.2 ml. of KOH is required. A precipitate ($KClO_4$) forms and is permitted to settle for 10 to 15 minutes in the cold or is removed by centrifugation.

One milliliter of the supernatant solution is placed in a quartz cuvette (3 ml. capacity), to which is added 1 ml. of buffer, 0.1 to 0.2 μM DPNH (about 0.05 to 0.10 ml. of DPNH solution as prepared),[11] and sufficient distilled water so that after the addition of enzyme the total volume is 3 ml. The optical density is read at 340 mμ in the spectrophotometer against a water blank and initially is approximately 0.300 to 0.600 O.D.

Lactic dehydrogenase is then added to the cuvette in an amount such that the reaction, as followed by O.D. change, is complete in from 4 to 7 minutes. The change generally consists of –0.040 to –0.085 O.D. unit. This requires about 3 to 5 μ Gm. of enzyme protein of the preparations used, determined by the method of Warburg and Christian.[19] One-hundredth milliliter of a 1 to 10 dilution of commercial enzyme may be employed. Dilution of concentrated or crystalline enzyme is made with cold 0.01 M NaCl.

An enzyme blank is run concomitantly, with the substitution of 1 ml. of water for the filtrate. The blank value generally is –0.001 to –0.002 O.D. unit in 7 minutes. Also, a pyruvate solution is analyzed as a check on the method. For this purpose dilute pyruvic acid or sodium pyruvate solutions may be used.

RESULTS AND DISCUSSION

Accuracy.—The method described is not dependent upon use of a pyruvate solution of known concentration either for construction of a standard curve or for estimation of results of individual analyses. The concentration of pyruvate is calculated* from the amount of DPNH entering into the reaction as determined optically, and from the molar extinction coefficient of DPNH,

*$\dfrac{[(\triangle O.D._{340\ m\mu}) - (\triangle O.D._{340\ m\mu}\ \text{blank})]\ cm^2}{6.22 \times 10^6\ cm^2\ mole^{-1}} \times$ mol. wt. pyruvate $\times$ dilution $\times 100 =$ mg. per 100 ml. of blood.

which is 6.22×10^6 cm^2 mole^{-1}.[8] There is a disadvantage in relying on a pyruvate standard, since purity of pyruvic acid or sodium pyruvate is frequently open to question.

Fig. 1 demonstrates the linear relationship between substrate concentration and optical density change over a range of pyruvate concentration greatly exceeding that encountered in normal and pathologic blood.

Recoveries of pyruvate added to whole blood in amounts equal to that normally present ranged from 97 to 104 per cent of theoretical. Duplicate determinations on blood filtrates showed an average difference of 3 per cent. However, the range of differences was 0 to 10 per cent, although in only two instances in 32 analyses did duplicates differ by more than 6 per cent.

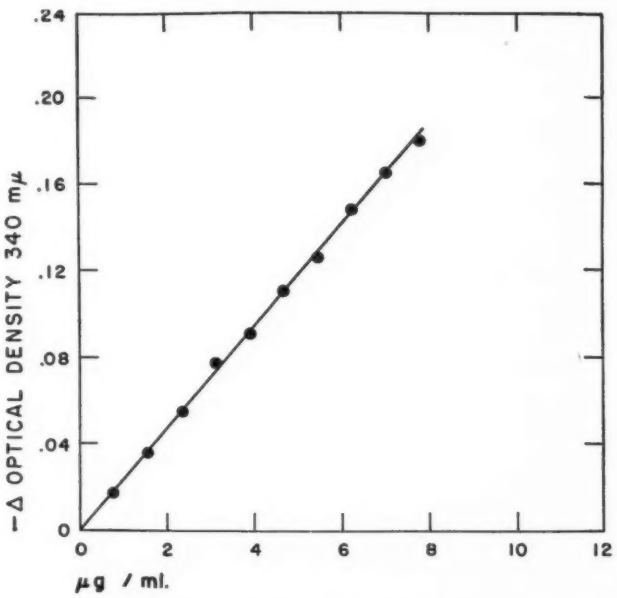


Fig. 1.—Graph of the decrease in the optical density vs. concentration of pyruvic acid in micrograms per milliliter. Solutions used were dilutions of a single standard whose pyruvate content was enzymatically assayed. The linearity between optical density change and substrate concentration is demonstrated.

Stability of Pyruvate in Perchloric Acid.—Numerous studies were carried out to determine the stability of pyruvate in perchloric acid. Blood pyruvate may stand at room temperature as the filtrate or as uncentrifuged precipitates for at least three hours without change. At 3° C. pyruvate shows no losses when kept for periods of time up to one month (Table I). Protein-free filtrates of blood prepared with cold trichloracetic acid gave values for pyruvate identical with those obtained on the same blood samples after perchlorate treatment when analyses were performed promptly. In the dinitrophenylhydrazine method of Friedemann and Haugen,[5] storage of the trichloracetic acid filtrates at 3° longer than 2 days is not recommended because of the generation of products which

TABLE I. STABILITY OF PYRUVIC ACID DURING STORAGE AND EXPERIMENTAL HANDLING

SAMPLE*	TREATMENT			PYRUVATE† MG./100 ML.
	STORAGE FORM, 3° C.	TIME STORED (DAYS)	OTHER	
1		0		0.68
1	Filtrate	1		0.66
2	Filtrate	1		0.66
3	Filtrate	2		0.67
4	Filtrate	15		0.63
5	Filtrate	44		0.63
6	Filtrate-precipitate mixture	1		0.66
7	Filtrate-precipitate mixture	2		0.66
8	Filtrate-precipitate mixture	15		0.66
9		0	Excess alkali in neutralization	0.67
10		0	30 min., standing room temp. in buffer pH 7	0.68

*Each sample is a separate protein precipitation of a 5 ml. aliquot of a single large blood specimen.

†All results agree within the observed experimental error of duplicates.

react with the reagent to give high values. No such limitation has been observed with the present method using perchloric acid filtrates. No studies of stability of pyruvate in trichloracetic acid filtrates were performed.

Other studies demonstrated that no loss of pyruvate occurred as a consequence of the addition of excess alkali in the filtrate neutralization step, provided the pH was immediately readjusted to less than pH 7. Also, cuvettes containing pyruvate, buffer, and DPNH at pH 7 may remain at room temperature for at least one-half hour before assay with no loss of pyruvate (Table I).

Specificity of the Determination.—Although Meister[14] points out that crystalline muscle lactic dehydrogenase may have other alpha keto acids as substrates, most of those studied were substances not known to occur in biologic material. Of those substrates of biologic occurrence, alpha ketoglutarate, phenylpyruvate, and oxaloacetate have substrate turnover rates so slow and enzyme affinities so low as not to interfere with accurate determination of pyruvic acid. Alpha ketoglutarate has the highest blood level of these possible substrates and is present in concentrations about $\frac{1}{5}$ those of pyruvate.[16] In prepared alpha ketoglutarate-pyruvate solution mixtures in which this ratio was increased to as much as 3:1, no interference with pyruvate determinations was observed (Table II).

In diabetic ketosis the presence of acetoacetate and large amounts of acetone in blood may interfere with phenylhydrazine methods for pyruvate analysis. Since the enzymatic spectrophotometric method of pyruvate analysis described above was developed for use in our studies of carbohydrate metabolism in diabetes, it was felt essential to evaluate possible interferences with the reaction caused by these ketone bodies. Meister[14] has shown that acetoacetate is not a substrate for lactic dehydrogenase. In the present study the addition of acetone to blood filtrate in levels of 800 mg. per cent and of dl-β-hydroxybutyrate in levels of 250 mg. per cent did not interfere with the pyruvate determination (Table II).

Normal Values in Man.—The morning blood pyruvate levels of 14 normal, fasting, resting subjects, 9 men and 5 women, are shown in Fig. 2. Two of the men had seven different determinations each. These results are included in the total of 26 assays recorded. The average blood pyruvate value was 0.66 mg. per cent with a range of 0.39 to 0.86 mg. per cent. These are in close agreement with those of other methods (Table III). It should be pointed out that there are marked differences in the normal range of pyruvate values reported by different investigators using the Friedemann and Haugen method. Kerppola[12] reports the normal range by this method to be 0.35 to 0.67 mg. per cent, much below the range of the original workers[5] (Table III), while the normal values reported by Frawley[2] appear to be much higher. The reasons for these discrepancies are not apparent.

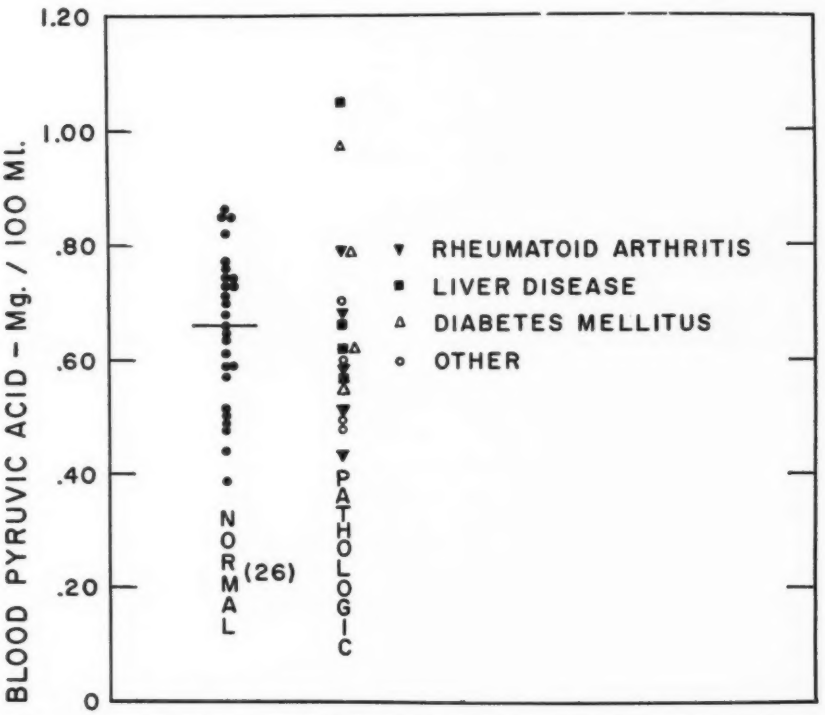


Fig. 2.—The range of blood pyruvic acid in twenty-six normal subjects and seventeen patients with disease.

Paired determinations done at intervals of 10 to 30 minutes on 10 fasting subjects showed considerable variation. In 5 subjects the levels were constant over these short intervals, but in the others differences ranging up to 18 per cent were noted. In two other fasting subjects tested at hourly intervals throughout the morning, declines of blood pyruvate of 27 and 30 per cent were observed, despite relative constancy of blood sugar levels.

Daily variations in the morning fasting blood pyruvate level were also detected. One normal man on whom seven determinations were done over an eight-week period had values ranging from 0.49 to 0.86 mg. per cent. These variations did not appear to be related to the technique of venepuncture, since,

TABLE II. SPECIFICITY OF THE PYRUVATE DETERMINATION WITH REGARD TO ALPHA KETOGLUTARATE, HYDROXYBUTYRATE, AND ACETONE

SOLUTION TESTED	ADDITION	PYRUVATE MG./100 ML.
Standard pyruvate	None	0.85
Standard pyruvate	Alpha ketoglutarate ratio $\frac{\text{ketoglutarate}}{\text{pyruvate}}$ 1:1	0.83
Standard pyruvate	Alpha ketoglutarate ratio 3.4:1	0.83
Blood	None	1.05
	Acetone 150 mg./100 ml.	1.05
Blood	None	0.44
	dl-β-hydroxybutyrate 250 mg./100 ml. acetone 800 mg./100 ml.	0.44

TABLE III. NORMAL BLOOD PYRUVATE LEVELS

METHOD	RANGE MG./100 ML.	AVERAGE MG./100 ML.
1. Lactic dehydrogenase	0.39 – 0.86	0.66
2. Dinitrophenylhydrazine, solvent extraction[5]	0.60 – 0.95	0.77
3. Dinitrophenylhydrazine paper chromatography[16]	0.26 – 0.77	0.54

in agreement with others,[3] we have found that blood stasis for two minutes caused by the use of a tourniquet, and indeed even the muscular exercise of 20 fist clenches, did not alter blood pyruvate values.

The method was also evaluated in subjects with diseases in which elevated pyruvate levels are known to occur. One of 4 patients with cirrhosis of the liver and 1 of 5 patients with well-controlled diabetes mellitus had elevated blood pyruvate levels of 1.05 and 0.97 mg. per cent, respectively. Elevated pyruvate values have been observed in these diseases by others.[18] Three patients with rheumatoid arthritis receiving maintenance doses of 15 to 20 mg. per day of Δ^1-cortisone (prednisone) and one receiving 3.5 mg. of Δ^1-9-α-fluorohydrocortisone had normal values. These are of interest in view of the elevation of blood pyruvate levels reported in subjects receiving large doses of cortisone,[6] hydrocortisone,[2] or ACTH.[12] Normal values were also observed in one case each of the following disorders: untreated rheumatoid arthritis, gout, parkinsonism, hyperthyroidism, and hypothryroidism.

SUMMARY

An enzymatic spectrophotometric method has been described for the determination of pyruvic acid in blood. The method is based upon measurement of the oxidation of reduced diphosphopyridine nucleotide by pyruvic acid in the presence of purified lactic dehydrogenase. This method is simple, accurate, sensitive, and highly specific. By this method the blood pyruvate level in fasting normal subjects ranges from 0.39 to 0.86 mg. per cent. Wide random variations in these levels may occur in the same individual over periods of time within one day or from day to day.

We wish to express our thanks to Dr. J. E. Seegmiller for suggesting the adaptation of an enzymatic assay to blood pyruvate analysis and to Dr. Bernard Horecker for a generous gift of purified lactic dehydrogenase.

REFERENCES

1. Cavallini, D., and Frontali, N.: Quantitative Determination of Keto-Acids by Paper Partition Chromatography, Biochim. et biophys. acta **13**: 439, 1954.
2. Frawley, T.: The Role of the Adrenal Cortex in Glucose and Pyruvic Acid Metabolism in Man Including the Use of Intravenous Hydrocortisone in Acute Hypoglycemia, Ann. New York Acad. Sc. **61**: 464, 1955.
3. Friedemann, T. E., and Haugen, G. E.: Pyruvic Acid. I. Collection of Blood for the Determination of Pyruvic and Lactic Acids, J. Biol. Chem. **144**: 67, 1942.
4. Friedemann, T. E., and Haugen, G. E.: Pyruvic Acid. II. The Determination of Keto Acids in Blood and Urine, J. Biol. Chem. **147**: 415, 1943.
5. Friedemann, T. E., Haugen, G. E., and Kmieciak, T. C.: Pyruvic Acid. III. The Level of Pyruvic and Lactic Acids and the Lactate-Pyruvic Ratio in the Blood of Human Subjects. The Effect of Food, Light Muscular Activity, and Anoxia at High Altitude, J. Biol. Chem. **157**: 673, 1945.
6. Gitelson, S.: The Effect of ACTH and Cortisone on the Blood Pyruvic Acid Level, Acta. endocrinol. **15**: 225, 1954.
7. Hess, B.: Bemerkungen zur Bestimmung der Benztraubensäure im menschlichen Serum, Klin. Wchnschr. **33**: 540, 1955.
8. Horecker, B. L., and Kornberg, A.: The Extinction Coefficients of the Reduced Band of Pyridine Nucleotides, J. Biol. Chem. **175**: 385, 1948.
9. Horecker, B. L., and Smyrniotis, P.: Phosphogluconic Acid Dehydrogenase From Yeast, J. Biol. Chem. **193**: 371, 1951.
10. Johannsmeier, K., Redetzki, H., and Pfleiderer, G.: Zur frage der beschleunigung des blutalkoholabbaus, Klin. Wchnschr. **32**: 560, 1954.
11. Kaplan, N. O., Colowick, S. P., and Neufeld, E. J.: Pyridine Nucleotide Transhydrogenase. II. Direct Evidence for and Mechanism of the Transhydrogenase Reaction, J. Biol. Chem. **195**: 107, 1952.
12. Kerppola, W.: Accumulation of Pyruvic Acid in Blood During Treatment With Cortisone or ACTH, and in Cushing's Syndrome, Acta. med. scandinav. **145**: 357, 1953.
13. Kornberg, A.: In Methods in Enzymology, Vol. 1, S. P. Colowick, and N. O. Kaplan, editors, New York 1955, Academic Press, Inc., p. 441.
14. Meister, A.: Reduction of α, γ-diketo and α-keto Acids Catalyzed by Muscle Preparation of Crystalline Lactic Dehydrogenase, J. Biol. Chem. **184**: 117, 1950.
15. Ochoa, S., Mehler, A. H., and Kornberg, A.: Biosynthesis of Dicarboxylic Acids by Carbon Dioxide Fixation. I. Isolation and Properties of an Enzyme From Pigeon Liver Catalyzing the Reversible Oxidative Decarboxylation of l-Malic Acid, J. Biol. Chem. **174**: 979, 1948.
16. Seligson, D., and Shapiro, B.: Alpha-Keto Acids in Blood and Urine Studied by Paper Chromatography, Anal. Chem. **24**: 754, 1952.
17. Seitz, W., Englhardt-Gölkel, A., and Schaffry, O.: Über eine fermentative bestimmungsmethode der α-ketoglutarsäure und ihre anwendung zur erforschung von Stoffwechselproblemen, Klin. Wchnschr. **33**: 228, 1955.
18. Smith, L. H., Ettinger, R. H., and Seligson, D.: A Comparison of the Metabolism of Fructose and Glucose in Hepatic Disease and Diabetes Mellitus, J. Clin. Invest. **32**: 273, 1953.
19. Warburg, O., and Christian, W.: Isolierung und kristallisation des Garungsferments Enolase, Biochem. Ztschr. **310**: 384, 1941.

A CLINICAL METHOD FOR THE ASSAY OF SERUM GLUTAMIC-OXALACETIC TRANSAMINASE

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CLINICAL experience in a number of laboratories has now shown that determination of the serum level of glutamic-oxalacetic transaminase (GOT)* can often be an extremely helpful diagnostic procedure. Clinical studies on GOT are most complete in relation to differential diagnosis of coronary artery disease,[6-9, 14, 15, 17] but it promises to be of value in several other clinical entities, including myocarditis and hepatocellular damage.[3, 10, 14, 19]

The properties of the serum enzyme so far studied are compatible with the hypothesis that it is identical with the tissue enzyme.[11]

A variety of methods for the assay of this enzyme have been reported in the biochemical literature.[1, 2, 4] However, most of these are too cumbersome or too time consuming for routine use in the clinical pathology laboratory. The present report describes a simple spectrophotometric procedure, modified from that published by Karmen,[5, 17] which can be carried out with use of a relatively inexpensive colorimeter and commercially available reagents.†

One technician can perform 20 to 30 assays daily and the reagents cost only a few cents per test. The assays are reproducible in our laboratory with a standard deviation of ± 6 per cent.

METHOD

Principle.—Glutamic-oxalacetic transaminase catalyzes the transfer of an amino group from aspartic acid to alpha-ketoglutaric acid, yielding oxalacetic acid and glutamic acid, or the reverse:

ASPARTIC ACID — ALPHA-KETOGLUTARIC ACID — OXALACETIC ACID — GLUTAMIC ACID

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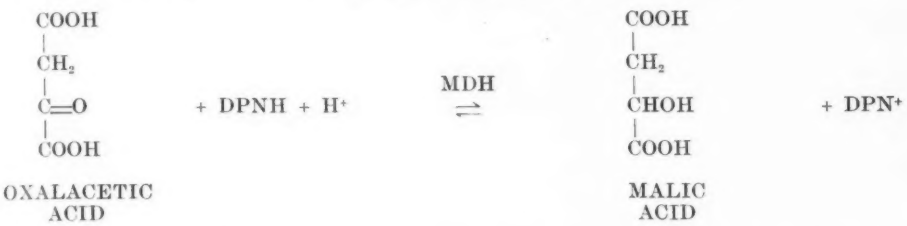
Received for publication Dec. 22, 1955.

*The following abbreviations have been used: glutamic-oxalacetic transaminase = GOT; malic dehydrogenase = MDH; diphosphopyridine nucleotide = DPN; reduced diphosphopyridine nucleotide = DPNH.

†The Bausch and Lomb Spectronic 20 colorimeter was used in most of these studies. As ordinarily supplied, the instrument reads only down to 350 mμ, but the manufacturer will select and specially calibrate instruments for use in this assay. The Beckman DU spectrophotometer affords somewhat greater precision. Of course, any spectrophotometer calibrated at 340 mμ can be used. All reagents are commercially available through the California Foundation for Biochemical Research, 3408 Fowler St., Los Angeles, Calif., or through the Sigma Chemical Company, 4648 Easton Ave., St. Louis, Mo.

This reaction is coupled with the malic dehydrogenase (MDH) reaction:



As fast as oxalacetic acid is formed in the GOT reaction, it is reduced to malic acid by the action of MDH, provided the concentrations of both MDH and DPNH are high enough. Under the specified conditions of substrate concentration, pH and temperature, then, the rate of disappearance of DPNH is directly proportional to the GOT concentration. The reaction is followed by observing the decrease in the characteristic light absorption peak of DPNH at 340 mμ where DPN has virtually no absorption.

Reagents.—

A. One-tenth molar potassium phosphate buffer, pH 7.4.—13.97 Gm. buffer quality anhydrous K_2HPO_4 + 2.69 Gm. buffer quality anhydrous KH_2PO_4 made up to 1 liter with water. Store in the cold.

B. Two-tenths molar L-aspartic acid, sodium salt.—2.662 Gm. L-aspartic acid dissolved in 70 ml. of solution A. Adjust to pH 7.4 (approximately 20 ml. 1 N NaOH). Dilute to 100 ml. with A. Keep frozen when not in use.

C. Reduced diphosphopyridine nucleotide (DPNH).—Available in different degrees of purity. If using 70 per cent pure DPNH, take up 10 mg. in 10 ml. of A. If using 90+ per cent pure DPNH, take up 7.5 mg. in 10 ml. of A. (When diluted 1:10 as in the assay procedure this solution should give an optical density (O.D.) about 0.5 at 340 mμ.) Keep frozen.

D. Malic dehydrogenase (MDH).—The enzyme is usually prepared from pig heart by the method of Straub.[18] The commercial preparations vary in potency. Enzyme should be diluted with solution A so as to contain 800 MDH units per milliliter. Diluted enzyme is less stable. Make up small amounts as needed. Refrigerate.

Saz[16] has used a simple procedure for obtaining MDH with minimal GOT activity from *Escherichia coli.**

E. One-tenth molar alpha-ketoglutaric acid, sodium salt.—1.47 Gm. alpha-ketoglutaric acid dissolved in 70 ml. of solution A. Adjust to pH 7.4 (approximately 20 ml. 1 N NaOH). Dilute to 100 ml. with solution A. Keep frozen.

Procedure.—

1. For each serum to be assayed, two Bausch and Lomb matched ½-inch test tubes† or two Beckman 1 cm. cuvettes are prepared as shown in Table I.

If, because of factors such as lipemia or bilirubinemia, the control tube cannot be balanced at zero, the assay is run with 0.1 ml. of serum or serum appropriately diluted with the phosphate buffer. Any changes in volume of serum or reagents used are compensated by changes in the phosphate buffer to keep the final volume (after step 3) at 3.0 ml.

2. The tube contents are thoroughly mixed and the tubes are allowed to stand for 30 minutes at room temperature. During this preincubation a variable amount of DPNH will

E. Coli (strain E 26) cells are ruptured sonically and centrifuged. The supernatant is fractionated with neutral ammonium sulfate, the fraction precipitating between 60 and 70 per cent saturation being taken. This is dialyzed against 4×10^{-2} M pH 7.5 phosphate buffer. Finally calcium phosphate gel pH 7.5, 0.75 Gm. per gram protein, is added. The supernatant after this gel step is used directly.

†Bausch and Lomb catalog, No. 33-29-27. Inside dimension 11.67 mm.; length 4 inches. Not all of these tubes, calibrated in the visible region, will match at 340 mμ. Tubes for the GOT assay should be matched at 340 mμ before use.

be oxidized, due to the presence in all sera of some intrinsic dehydrogenase activity together with small amounts of appropriate substrate (Fig. 1). In most cases there is ample residual DPNH after this preincubation, but if the optical density has fallen below 0.200 an additional 0.1 ml. of DPNH is added (Fig. 1).

3. After 30 minutes the tubes are brought to the temperature chosen for the assay, 0.2 ml. of 0.1 M alpha-ketoglutarate is added to each tube, and the contents are again thoroughly mixed.

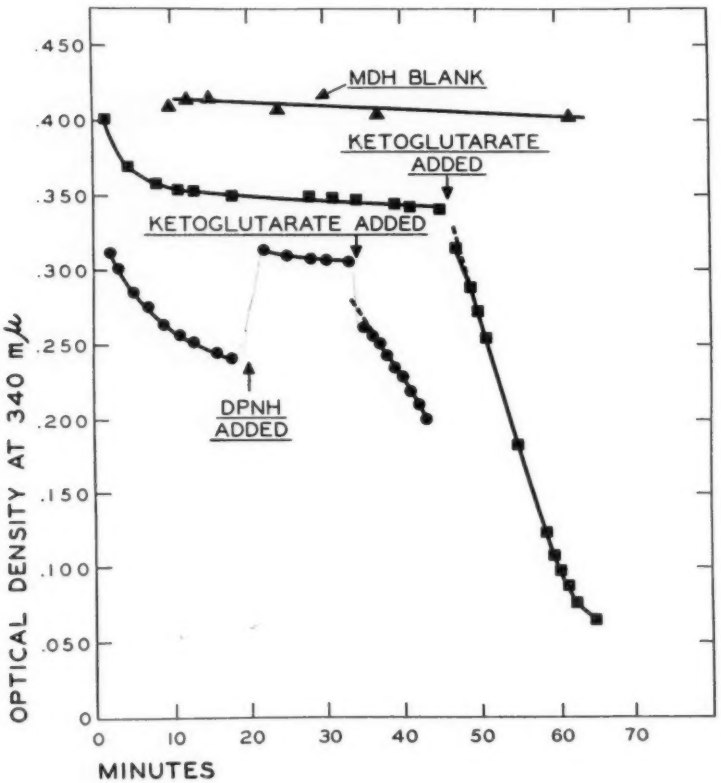


Fig. 1.—Upper curve: Intrinsic GOT activity of MDH. Lower curves: Typical assay curves showing DPNH oxidation during 30-minute preincubation period. Note less than maximal rate during first minute or two after addition of ketoglutarate and less than maximal rate when DPNH levels fall to low values.

4. Two minutes after the addition of alpha-ketoglutarate, readings at 340 mμ are begun. With use of the control tube as a blank, the optical density of the assay tube is read exactly 2, 4, 6, 10, and 15 minutes after the addition of alpha-ketoglutarate. For samples with very low GOT activity, additional readings may be taken at 20, 25, and 30 minutes. The reaction continues linearly for at least an hour, provided the DPNH concentration does not fall below 0.200. With samples of high GOT activity the optical density may fall below 0.200 before the 15-minute reading. To obtain accurate values for such samples, one should repeat the test using less serum or serum diluted with phosphate buffer.

5. From the rate of change in O.D. at 340 mμ, corrected as discussed below, the GOT concentration of the serum is directly calculated.

TABLE I

	CONTROL TUBE I	ASSAY TUBE II
0.2 M Aspartate	0.5 ml.	0.5 ml.
MDH (800 u/ml.)	0.1 ml.	0.1 ml.
DPNH (0.75 to 1.00 mg./ml.) (see above)		0.3 ml.
Serum	0.2 ml.	0.2 ml.
0.1 M Phosphate buffer, pH 7.4	2.0 ml.	1.7 ml.

DISCUSSION

Temperature.—As with all enzymatic reactions the rate of this coupled reaction varies markedly with temperature. The magnitude of the effect is shown by Fig. 2. For maximum accuracy and reproducibility, tubes should be incubated in a constant temperature bath during the reaction and removed only briefly for readings. Since most clinical laboratories have available a 37° C. water bath, we suggest that all assays be run at that controlled temperature.

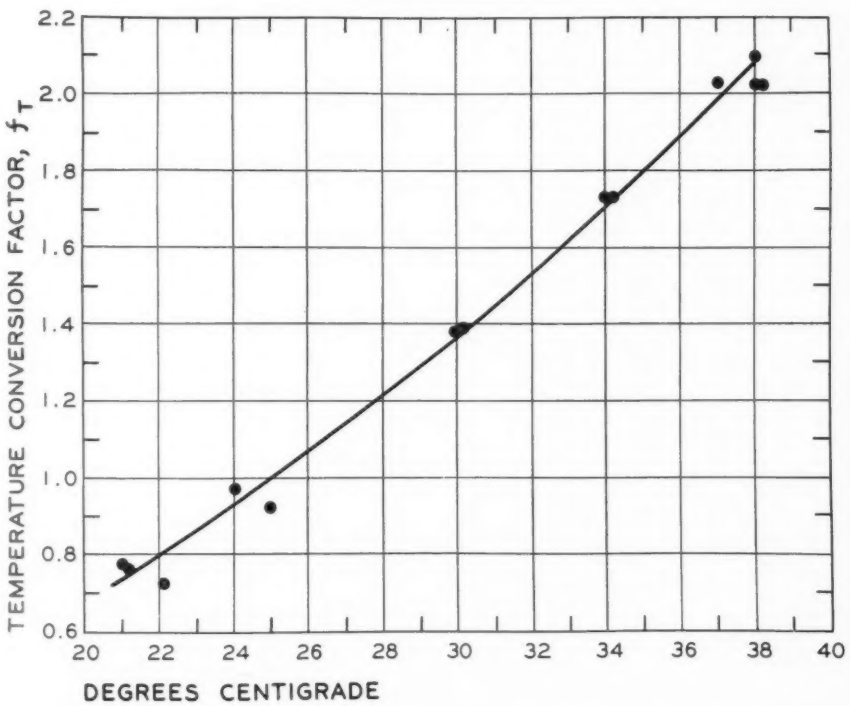


Fig. 2.—Temperature conversion factors for GOT assay. Observed value at any temperature is divided by f_T to obtain corresponding value at 25° C. The experimental points correspond to observed GOT activities ranging from about 40 up to 120 units per milliliter at the different temperatures used.

On the other hand, provided room temperature is sufficiently constant, it is possible to obtain accurate and reproducible results incubating the tubes in room air. Working in an air-conditioned building, we have found this quite satisfactory for clinical purposes.

The temperature effect is reproducible under the prescribed conditions, and thus results obtained at any temperature can be readily converted to a chosen standard temperature. Since most of the results reported to date have been obtained at 25° C., we suggest that results be converted to that temperature for the sake of uniformity in reporting. Values obtained at a given temperature are divided by the temperature conversion factor, f_T, taken from Fig. 2.

pH.—The serum enzyme shows a pH optimum at about 7.4, close to that reported by Cohen for the enzyme in pig heart.[2] The reaction rate falls off rapidly on either side of the optimum and so the pH of the reagents should be adjusted precisely.

Substrate Concentration.—The final concentrations of aspartic acid and alpha-ketoglutaric acid represent saturation concentrations for the transaminase reaction. Further increases in substrate concentration do not significantly increase the rate of the reaction. Similarly the final concentrations of DPNH and of MDH are such that further increases do not significantly increase the over-all rate of the reaction under the conditions described, at least when the serum GOT concentration is less than 200 units per milliliter. With these concentrations of DPNH and MDH, the GOT reaction is rate limiting and the DPNH disappearance rate reflects the rate of the GOT reaction. The DPNH disappearance after the first 2 minutes following the addition of alpha-ketoglutarate should be strictly linear. If for any reason the effective concentration of the reactants is too low, this will generally be reflected in a nonlinear rate. For example, when the DPNH has been largely consumed, its concentration will no longer be high enough to maintain a maximal rate of reaction and the curve for DPNH disappearance will fall off as shown in Fig. 1.

At the very beginning of the reaction, just after the addition of alpha-ketoglutarate, the DPNH disappearance rate may be less than maximal, probably because a certain minimum oxalacetic acid concentration is required for maximum rate. For this reason readings are not begun until two minutes after the addition of alpha-ketoglutarate.

MDH Blank.—Preparations of malic dehydrogenase generally contain small but measurable amounts of GOT activity. The level of this GOT activity should be checked for each batch of MDH. The assay is run in the usual manner, substituting phosphate buffer for serum, and the observed rate of fall in optical density at 340 mμ is subtracted from the observed rates in all assays. Commercial preparations of MDH should show a rate of DPNH disappearance less than 0.001 per minute. If the MDH blank is less than 0.0005 per minute it can be neglected for most purposes.

Potency of MDH Preparations.—Activity of the MDH preparation is tested at least once a month. The activity in MDH units per milliliter can be directly determined,[12] or, more conveniently, the potency can be validated by showing that the reaction rate is strictly proportional to GOT concentration. Graded aliquots of the same serum sample are assayed on the same day. The corrected

rates of DPNH disappearance should be in direct proportion to the size of serum aliquot used. For example, a serum with 50 to 100 units of GOT per milliliter can be assayed using 0.1, 0.2, and 0.3 ml. of serum in separate runs.

It should be noted that most sera contain some intrinsic MDH activity. Even when no MDH is added, there is an appreciable rate of DPNH oxidation in the GOT assay. For this reason omission of MDH does not provide a true blank for the assay.

Standardization and Reproducibility.—A reference standard of the GOT enzyme is not at present available. However, if the following two criteria, discussed in preceding paragraphs, are met it can be concluded that the assay is working properly.

1. *The curve for DPNH disappearance is strictly linear in all assays.*
2. *The rate of the reaction is directly proportional to GOT concentration.*

Ten replicate assays on the same serum sample gave a mean value of 48.8 ± 3.0 units per milliliter, a standard deviation of ± 6 per cent. The range was from 44.4 to 52.9 units per milliliter. Values obtained using the Bausch and Lomb Spectronic 20 colorimeter checked the values obtained simultaneously on the Beckman to within 10 per cent.

Calculations.—One unit of GOT is the amount of enzyme that will cause the reading at 340 mμ to decrease at the rate of 0.001 O.D. units per minute per centimeter light path at 25° C. under the described conditions. This definition is essentially that proposed by Karmen[5] modified by specification of temperature and light path. Using the conversion factors from Fig. 2, results obtained at any temperature are converted to 25° C. The specification of light path is equivalent to defining the unit in terms of micromoles DPNH oxidized per minute. When the effective light path is not 1 cm., the appropriate conversion factor is easily determined. For the Bausch and Lomb ½-inch matched tubes the *effective* light path is 0.83 cm.

Before any assay results are accepted for calculation, the linearity of the reaction rate is confirmed by inspection or plotting of the readings. Within the limits of error for reading the instrument, the rate of change in O.D. should remain constant during the assay. If the rate is linear, the difference between any two readings can be used in calculating results. Errors due to instrument instability, reading errors, etc., are minimized by using the first and the last readings.

$$\text{GOT (u/ml.)} = \left[\frac{\text{O.D.}_1 - \text{O.D.}_2}{t_2 - t_1}\right] \times \frac{1}{\text{ml. serum}} \times \frac{1}{f_T} \times \frac{1}{f_P} \times 1{,}000$$

where:

O.D._2 = optical density at time t_2
O.D._1 = optical density at time t_1
f_T = temperature conversion factor
f_P = effective light path = 0.83 for the Bausch and Lomb Spectronic 20.

When the MDH blank is appreciable (see above) it is subtracted from the first term in the above equation.

Properties of Serum GOT.—GOT in serum is stable at refrigerator temperatures, showing little or no decrease in activity for 3 weeks. After 4 months at 4° C., serum still retains about 60 per cent of its initial GOT activity. Freezing does not destroy the enzyme.

The GOT reaction requires pyridoxal phosphate as a coenzyme.[13] However, the enzyme as it occurs in serum appears to be saturated with coenzyme, since the addition of 300 μ Gm. per milliliter of pyridoxal phosphate did not increase the observed GOT activity.

Normal red blood cells contain GOT at a much higher concentration than normal serum.[6, 17] Hemolysis must be avoided during collection of samples. There does not appear to be any significant difference between GOT concentrations in serum and plasma. Heparin and oxalate do not interfere with the assay.

Normal Values.—The mean value for 20 young normal adults in our series was 16 units per milliliter with a range from 10 to 33 units per milliliter.

Repeated determinations on a young man showed no significant changes through the day (9:30 A.M., 18.2; 11:30 A.M., 18.4; 2:30 P.M., 17.0 per milliliter). Apparently fasting samples are not obligatory, although small effects have not been ruled out. Lipemia, if it is not so marked as to interfere with the readings, does not alter the levels. After a 100 Gm. fat meal the following values were found: control, 32; 2 hours, 32; 4 hours, 28; 6 hours, 32 units per milliliter.

SUMMARY

1. A relatively simple and inexpensive spectrophotometric assay procedure for serum glutamic-oxalacetic transaminase is described. The assay procedure lends itself to routine use in the clinical laboratory.

2. Standardization in the assay and in reporting of enzyme levels is proposed.

3. Some properties of the enzyme in normal serum are described.

REFERENCES

1. Awapara, J., and Seale, B.: Distribution of Transaminases in Rat Organs, J. Biol. Chem. **194**: 497-502, 1952.
2. Cohen, P. P.: Transamination With Purified Enzyme Preparations, J. Biol. Chem. 136: 565-584, 1940.
3. Glassner, H. F., Jacobs, H. I., and Agress, C. M.: Serum Transaminase Levels in Experimental Pulmonary Infarction and Pericarditis, Circulation **12**: 712, 1955.
4. Green, D. E., Leloir, L. F., and Nocito, V.: Transaminases, J. Biol. Chem. **161**: 559-582, 1945.
5. Karmen, A.: A Note on the Spectrophotometric Assay of Glutamic-Oxalacetic Transaminase in Human Blood Serum, J. Clin. Invest. **34**: 131-133, 1955.
6. Karmen, A., Wroblewski, F., and LaDue, J. S.: Transaminase Activity in Human Blood, J. Clin. Invest. **34**: 126-131, 1955.
7. Kattus, A. A., Jr., Semenson, C., and Watanabe, R.: Use of Serum Transaminase in Detection of Myocardial Infarction in Uncertain Cases, Circulation **12**: 729, 1955.
8. LaDue, J. S., Wroblewski, F., and Karmen, A.: Serum Glutamic Oxalacetic Transaminase Activity in Human Acute Transmural Myocardial Infarction, Science **120**: 497-499, 1954.
9. LaDue, J. S., and Wroblewski, F.: The Significance of the Serum Glutamic Oxalacetic Transaminase Activity Following Acute Myocardial Infarction, Circulation **11**: 871-877, 1955.

10. Nydick, I., Tang, J., Stollerman, G. H., Wroblewski, F., and LaDue, J. S.: The Influence of Rheumatic Fever on Serum Concentrations of the Enzyme, Glutamic Oxalacetic Transaminase, Circulation **12**: 795-806, 1955.
11. Nydick, I., Wroblewski, F., and LaDue, J. S.: Evidence for Increased Serum Glutamic Oxalacetic Transaminase Activity Following Graded Myocardial Infarcts in Dogs, Circulation **12**: 161-168, 1955.
12. Ochoa, S.: Malic Dehydrogenase From Pig Heart, Methods in Enzymology, Vol. 1, New York, 1955, Academic Press, Inc., pp. 735-739.
13. O'Kane, D. E., and Gunsalus, I. C.: The Resolution and Purification of Glutamic-Aspartic Transaminase, J. Biol. Chem. **170**: 425-432, 1947.
14. Ostrow, B. H., Steinberg, D., Ticktin, H. E., and Evans, J. M.: Serum Transaminase: A Clinical and Electrocardiographic Correlation in Patients With Suspected Myocardial Infarction, Circulation **12**: 756, 1955.
15. Ostrow, B. H., Steinberg, D., Ticktin, H. E., and Evans, J. M.: Serum Glutamic-Oxalacetic Transaminase in the Differential Diagnosis of Coronary Artery Disease, A Report on 200 Cases. To be published.
16. Saz, A. K.: Personal communication.
17. Steinberg, D., and Ostrow, B. H.: Serum Transaminase as a Measure of Myocardial Necrosis, Proc. Soc. Exper. Biol. & Med. **89**: 31-34, 1955.
18. Straub, F. B.: Purification of Malic Dehydrogenase, Ztschr. f. physiol. Chem. **275**: 63-72, 1942.
19. Wroblewski, F., and LaDue, J. S.: Serum Glutamic Oxalacetic Transaminase as an Index of Liver Destruction and Disease, Clin. Res. Proc. **3**: 49, 1955.

TECHNIQUE FOR PERMANENT LONG-TERM INTUBATION OF RAT AORTA

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INTRODUCTION

A TECHNIQUE has been developed whereby a small polyethylene tube is permanently implanted in the aorta of a rat. The free end of the tube is exteriorized at the base of the neck and sealed so that continuous access to the aorta may then be assured without anesthesia and without painful stimulation of the rat. Three general kinds of procedures have been carried out with this preparation.

1. Injections of various materials (hormones, drugs, and India ink) have been made into the aorta and their effects and distribution studied in the tissues.[1]

2. Blood pressure has been measured directly through such tubes.[2]

3. Blood samples have been withdrawn at various intervals through the tube (Fig. 1).

OPERATIVE PROCEDURE

Under ether anesthesia the abdomen is opened, and the abdominal aorta is exposed at the bifurcation of the iliacs. The peritoneal layer and connective tissue overlying the aorta are pushed away with a dry sponge so that the aorta lies free of any extraneous tissue. A soft clamp is then placed on the aorta about 2½ to 3 cm. above the bifurcation. With use of a straight-cutting-edge needle, a small hole is then made in the aorta, and the polyethylene tube (PE No. 10, Clay-Adams) is inserted through the hole for a distance usually of about 2 cm. (longer distances are possible). The tube is marked prior to insertion to control the depth of insertion. The clamp on the aorta is then removed. An anchor suture is placed around the tube near its point of emergence from the aorta and this suture is fastened to the deep back muscles in this area. The tube is then made to follow the contour of the pelvic floor and brought out of the abdomen. The tube is again anchored to the belly muscles at the lower end of the incision. The abdominal viscera are then replaced and the abdominal muscles closed. The free end of the tube (which is filled with normal saline and clamped at the free end before surgery begins) is then carried under the skin to a point at the dorsal base of the neck. Here a small incision in the skin permits the exteriorization of the tube. The abdominal skin is then closed and a small amount of normal saline is injected through the tube to evacuate any blood which may have entered. The tube is then cut off to the desired length, keeping it clamped (with a shod clamp) meanwhile. The end of the tube is then sealed by clamping with a smooth warmed clamp. Thirty thousand units of penicillin are injected intramuscularly and the animal is returned to its cage. The procedure requires about 30 minutes' time. A recovery period of 5 days after the operation is allowed before the animal is used for experimental purposes.

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Received for publication Jan. 6, 1956.

The outside diameter of the tube has a cross-sectional area approximately 25 per cent of the cross-sectional area of the lumen of the aorta. This degree of reduction in the cross-sectional area of the aorta has no deleterious effect on the function of the hind limbs. If the tube is completely filled with saline and properly sealed, no appreciable amount of blood enters it and no clotting occurs in the tube.

GENERAL EXPERIENCE AND OBSERVATIONS

1. *Injections.*—The preparation was developed, in the first instance, to permit us to inject ink into the kidneys of rats under more physiologic conditions than most other injection techniques in small animals permit. By injecting the ink rapidly (in 3 or 4 seconds) and guillotining the rats immediately between heart and kidney, we obtained an outline of the blood flow pattern during the brief 3- or 4-second injection period. The picture of tissue sections thus obtained might be compared to a single frame of a motion picture. Strikingly different types of patterns were demonstrated.[1]

2. *Blood Pressure.*—Blood pressure was measured directly.[2] Though we have not done it, we felt that it would be possible to develop a bell-jar arrangement which would permit the rat to move freely within the jar while his blood pressure was being continuously recorded. By placing a 2-way valve in the tube system, one also could inject drugs into the animal without handling it and with only a brief interruption of the pressure record.

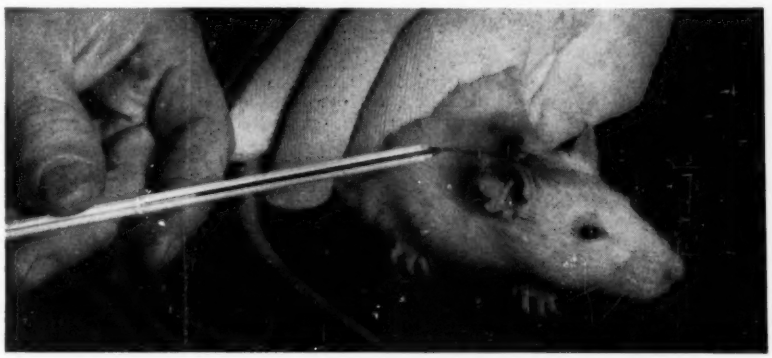

Fig. 1.—Collecting blood sample in viscometer tube.

3. *Blood Samples.*—A number of experiments have been carried out which involve taking several samples at time intervals extending over a period as long as two weeks (Fig. 1).

4. *Duration of Preparation.*—We retired about 35 such animals from experimental use about 10 months ago. Except for 2 or 3 spontaneous deaths and 6 or 7 which were killed for examination from time to time, all are still alive and appear to be in good health. At this time they are about 18 months old. Gross inspection of the aorta, adrenals, and other abdominal viscera of the animals killed has revealed no evidence of pathology in them. Those that died were cannibalized and could not be examined.

5. *Aneurisms.*—In our earlier use of the preparation we lost quite a few animals because they developed aneurisms at about the fourteenth postopera-

tive day, plus or minus 5 or 6 days. By using a softer clamp on the aorta during surgery, we have reduced this incidence to a small value, perhaps in the neighborhood of 5 per cent at present. We have generally used rats from 3 to 6 months of age but have used a few rats which were 12 to 15 months old when intubated. None of these older rats have developed aneurisms. It is our impression that the incidence of aneurism is in inverse proportion to the age of the animal at time of surgery. We have recently found that by gently heating the tubing it is possible to stretch it out and thereby reduce the stiffness of the end of the tube. It is our impression that this reduces the trauma to the aorta from the whipping action of the tube. This may be of value in further reducing the aneurism problem.

6. *Maintenance of Animals.*—An animal so prepared with a tube emerging from the back of his neck must be caged separately so that other rats will not chew its tube off. Even when the rats are caged individually the tube should protrude as little as possible to minimize the possibility of the rat reaching his own tube and pulling the end into his mouth. Of course the length of exposed tube required should vary with the number of times one plans to use it, because a few millimeters of tube are lost each time it is unsealed.

7. *Other Values of the Preparation.*—Besides the advantage of avoiding anesthesia and pain, this preparation has the further advantage that it permits a "before" and "after" type of experiment design in many cases. It also makes it possible to use rats for many kinds of experiments which formerly could be done only in larger animals.

REFERENCES

1. Still, Joseph W., and Whitcomb, Ernest R.: An Investigation of Renal Shunts in Rats, Am. J. Physiol. **178**: 399, 1954.
2. Still, Joseph W., Pradhans, Sachindra N., and Whitcomb, Ernest R.: Direct Measurements of Aortic Blood Pressure in Unanesthetized Rats, J. Appl. Physiol. **8**: 875, 1956.

THE EVALUATION OF SEROLOGIC TESTS FOR LEPTOSPIROSIS

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IN A previous paper[1] a method for preparing "particulate" suspensions of Leptospira for use as complement-fixing antigens was described. Although the experimental data were concerned primarily with the cross reactions obtained with antigens prepared from seven Leptospira serotypes and the sera of rabbits hyperimmunized with these serotypes, data also were obtained on a limited number of human, bovine, and canine sera from cases of leptospirosis.

The present paper reports additional studies on human and animal sera from cases of leptospirosis, sera from normal individuals and various animals, and sera from cases of infections due to other agents. Some sera also were tested by the capillary agglutination and microscopic agglutination techniques.

MATERIAL AND METHODS

Antigens.—The three Leptospira serotypes *(L. icterohemorrhagiae, L. canicola,* and *L. pomona)* used for preparation of the capillary agglutination and complement-fixing antigens were grown on a modified Schüffner's medium by the simplified procedure reported previously.[2] The cultures were incubated at 28° C. for 7 days, centrifuged at 20,000 g for 20 minutes, and the organisms concentrated by resuspension at one-tenth the original volume in phosphate-buffered saline, pH 7.0, containing 10 per cent formaldehyde. After standing for 48 hours at 5° C., the suspensions were centrifuged at 20,000 g for 10 minutes, and, with a minimum of agitation, the Leptospira were transferred by means of an applicator stick to a graduated centrifuge tube containing approximately 10 ml. of barbital saline. The organisms were then centrifuged in a 15 ml. graduated centrifuge tube at 2,500 r.p.m. (900 g) for 20 minutes and, after the measurement of the sediment, were resuspended in sufficient barbital saline (containing merthiolate 1 in 10,000) to yield a 1.25 per cent suspension. The suspensions were homogenized by repeated aspirations with a 20 ml. syringe equipped with a 3-inch, 20-gauge needle.

The capillary antigens for the three serotypes were prepared by repeated centrifugation as described by Stoenner.[3]

L. icterohemorrhagiae and *L. pomona* "sonic" antigens were obtained through the courtesy of the Veterinary Division of the Army Medical Service Graduate School.

Sera.—The following groups of sera were tested:

1. Sera from apparently normal humans, cows, dogs, and goats. The normal human sera were obtained from a group of healthy young adults recently commissioned in the U. S. Public Health Service. The goat sera were obtained from healthy stock animals prior to immunization. The dog sera were procured from the Virus and Rickettsia Labora-

From the Communicable Disease Center, Public Health Service, Department of Health, Education, and Welfare, Chamblee, Ga.
Received for publication Jan. 13, 1956.

tory, Communicable Disease Center, where the animals were kept as controls for other studies. Through the courtesy of Mr. Frank Turnipseed, Louisiana Experiment Station, Baton Rouge, specimens of bovine sera were obtained from veterinary-inspected herds.

2. Sera from patients with syphilis, having Kahn titers of 1 to 8 or above, were obtained through the courtesy of Dr. Sunkes, Georgia Department of Health Laboratories.

3. Sera from cattle giving positive agglutination tests for Brucella were obtained from Mr. Turnipseed, Louisiana Experiment Station, Baton Rouge.

4. Sera from patients with infectious hepatitis and homologous serum jaundice were obtained through the courtesy of Dr. Gerald Cooper, Hematology and Biochemistry Section, Communicable Disease Center.

5. Sera from human, bovine, and canine leptospirosis were obtained from various sources.

Complement Fixation.—

Five 50 per cent unit technique: Each serum, after inactivation, was diluted 1 in 8 in barbital-buffered saline, and a series of tubes containing 0.25 ml. quantities of progressively doubled dilutions was prepared. Appropriately diluted antigens in 0.25 ml. amounts were added to each tube as was five 50 per cent units of complement in 0.5 ml. of the buffer solution. After fixation at 4 to 6° C. for 15 to 18 hours, the tubes were placed at room temperature while the sensitized cells were prepared. The sensitized cells in 0.5 ml. amounts (0.25 ml. of a 2 per cent suspension of washed sheep cells and 0.25 ml. of hemolysin diluted to contain 4 units) were added to all tubes, and readings were made after 30 minutes' incubation in a 37° C. water bath. Titers were recorded as the highest dilution of serum giving a 3-plus or 4-plus reaction (30 per cent hemolysis or less).

The anticomplementary titer of each antigen was determined by testing with 1.25, 2.5, and 5.0 units of complement. The greatest dilution of antigen yielding 90 per cent hemolysis or less with two and five-tenths 50 per cent units was considered to be anticomplementary. The antigen dilution used in the tests for serum antibody was twice that showing anticomplementary activity.

Kolmer technique: A modified (half volume) Kolmer procedure[4] was used for comparisons of results obtained with the "sonic" and the "particulate" antigens. The dilutions of the particulate antigens used in the Kolmer complement fixation tests were determined by preliminary titrations of the anticomplementary properties using Kolmer units. The sonic antigen was diluted 1 in 4, as recommended by the Army Medical Service Graduate School.

Capillary agglutination technique: The capillary agglutination tests were set up as described by Stoenner,[3] employing progressively doubled dilutions of serum beginning with 1 in 8. A positive test was denoted when definite agglutination occurred in the capillary tube after incubation at 37° C. for 20 hours, followed by refrigeration at 4° C. for 1 hour.

RESULTS

Comparison of Titers Obtained With the "Sonic" and "Particulate" Antigens.—In Table I are presented the results of complement fixation tests, using the sonic antigens (Kolmer technique) and the particulate antigens (Kolmer and five 50 per cent unit techniques) as obtained on sera from 5 human, 4 bovine, and 2 canine cases of leptospirosis. The data indicated that, with the same complement fixation technique, the sonic antigens were less sensitive than the particulate antigens, particularly the *L. icterohemorrhagiae* antigens which gave negative results on all but one serum in the lowest dilution of serum tested (1 in 8). The differences between the titers obtained by the sonic and particulate antigens were not as marked with the bovine sera tested with *L. pomona.*

In general, the Kolmer technique gave lower titers with the particulate antigens than were obtained with the procedure employing five 50 per cent units of complement. Exceptions were noted, however; the reverse situation occurred in human serum 3,717 tested with *L. canicola* and *L. pomona* antigens; in human serum 939 tested with the *L. canicola* antigen; in bovine sera 3,525, 2,156 tested with the *L. canicola* antigen; and in canine serum 3,884 tested with *L. pomona.* Although isolations of Leptospira were not made, making impossible serotype identifications of the infecting strains, it is noted that the exceptions (higher titers with the Kolmer technique) occurred in tests made with antigens other than the serotype giving the highest titers. In the case of the *L. pomona* antigen, it should be noted that the dosages of antigen used in the two tests were not the same, since the anticomplementary titers as determined by the two methods were different. Other factors which may be concerned include the quantitative differences in the amounts of reagent used.

TABLE I. COMPLEMENT FIXATION TITERS ON SERA FROM CASES OF LEPTOSPIROSIS USING 3 LEPTOSPIRA SEROTYPE ANTIGENS, 2 TYPES OF ANTIGEN PREPARATION (SONIC AND PARTICULATE), AND 2 FIXATION TECHNIQUES

SEROTYPE		L. ICTEROHEMORRHAGIAE			L. CANICOLA		L. POMONA		
TYPE OF ANTIGEN		SONIC	PARTICULATE		PARTICULATE		SONIC	PARTICULATE	
UNITS OF COMP.		2 FULL UNITS	2 FULL UNITS	5-50% UNITS	2 FULL UNITS	5-50% UNITS	2 FULL UNITS	2 FULL UNITS	5-50% UNITS
ANTIGEN	DILUTION	1-4	1-32	1-32	1-32	1-32	1-4	1-32	1-8
Source of serum	Human 1,317	Neg.*	1-16	1-64	Neg.	Neg.	Neg.	1-8	1-16
	Human 669	Neg.	1-32	1-128	Neg.	Neg.	Neg.	Neg.	Neg.
	Human 939	Neg.	1-128	1-256	1-8	Neg.	Neg.	1-32	1-128
	Human 851	1-8	1-16	1-32	1-16	1-32	1-16	1-32	1-128
	Human 3,717	Neg.	1-64	1-128	1-16	Neg.	Neg.	1-32	Neg.
	Bovine 3,525	Neg.	Neg.	Neg.	1-8	Neg.	1-16	1-16	1-128
	Bovine 3,529	Neg.	Neg.	Neg.	1-8	1-16	1-8	1-8	1-32
	Bovine 2,355	Neg.	1-8	1-32	1-16	1-64	1-16	1-32	1-128
	Bovine 2,156	Neg.	1-8	1-32	1-64	1-32	1-64	1-128	1-1,024
	Canine 3,884	Neg.	1-32	1-64	1-64	1-256	Neg.	1-32	1-16
	Canine 3,046	Neg.	Neg.	Neg.	1-16	1-128	Neg.	Neg.	Neg.

*Neg. = No fixation in serum diluted 1 in 8.

Titers Obtained With Sera From Normal Men and Animals.—Since the technique employing the particulate antigens and five 50 per cent units of complement technique appeared to be the most sensitive method of detecting antibodies in sera from leptospirosis, the procedure was used to test three antigens (*L. icterohemorrhagiae, L. pomona,* and *L. canicola*) with sera from 33 normal humans, 10 normal dogs, and 101 normal cows. The results, presented in Table II, showed that none of the humans and goats gave a positive reaction with any of the three antigens. On the other hand, about one-fourth of the canine sera reacted in low titer with the *L. icterohemorrhagiae* and *L. canicola* antigens, and about 40 per cent reacted with the *L. pomona* antigen. Ten per cent of the sera from normal cows reacted with the *L. pomona.*

Titers Obtained on Serum From Infections Due to Other Organisms.—Sera from 11 patients with homologous serum jaundice, from 10 patients with in-

fectious hepatitis, from 39 Kahn-positive patients with syphilis, and from 48 patients with tuberculosis were tested with the *L. icterohemorrhagiae, L. canicola,* and *L. pomona* antigens, all giving negative results in the lowest dilution (1 in 8) tested.

TABLE II. COMPLEMENT FIXATION REACTIONS ON SERA FROM APPARENTLY NORMAL ANIMALS

ANTIGEN	SERUM TITERS	SOURCE OF SERUM							
		HUMAN		CAPRINE		CANINE		BOVINE	
		NO.*	%	NO.	%	NO.	%	NO.	%
L. icterohemorrhagiae	Neg.†	33	100	10	100	22	76	98	97
	1-8	0	0	0	0	2	24	3	3
	1-16	0		0		5		0	
	Total	33	100	10	100	29	100	101	100
L. canicola	Neg.	33	100	10	100	22	76	97	96
	1-8	0	0	0	0	4	24	4	4
	1-16	0		0		3		0	
	Total	33	100	10	100	29	100	101	100
L. pomona	Neg.	33	100	10	100	18	62	91	90
	1-8	0	0	0	0	2	38	5	10
	1-16	0		0		7		4	
	1-32	0		0		2		1	
	Total	33	100	10	100	29	100	101	100

*No. = Number of sera.
†Neg. = No fixation in serum diluted 1 in 8.

Complement fixation tests with *L. pomona* antigen were performed on sera from 78 cows showing positive agglutination reactions with *Brucella.* Two sera were positive in a 1 in 16 dilution and three in a 1 in 8 dilution. None of the remaining 73 sera in the 1 in 8 dilution fixed complement.

Capillary Agglutination Titers on Sera From Apparently Normal Humans, Dogs, and Cows.—The data presented in Table III were obtained on groups of sera differing from those given in Table II. The results of the capillary agglutination test are in the same ranges as were observed in the complement fixation test. About one-third of the bovine sera reacted with the *L. pomona* antigen and a much lower percentage with the *L. icterohemorrhagiae.* Similarly one-third of the canine sera reacted with *L. pomona* antigen, whereas about one-eighth of the sera reacted with *L. icterohemorrhagiae* and *L. canicola* antigen. Positive capillary agglutination tests with normal human sera were exceptional.

Comparison of Complement Fixation, Capillary Agglutination, and Microscopic Agglutination Titers on Sera From Humans, Cows, and Dogs With Leptospirosis.—The results, presented in Table IV, showed no consistent agreement between the titers obtained by the different techniques and with the three antigens. It is unfortunate that no data were available concerning the serotype causing each infection. However, if it is assumed that the serotype

giving highest titers "across the board" with all three tests is the serotype responsible for the infection, it becomes apparent that the irregularities are observed only in tests performed with the heterologous antigens. On this basis it is assumed that human sera 3,717, 1,448, and 3,884 were from patients with *L. icterohemorrhagiae* infections, and sera 699 and 1,315 were from *L. pomona* infections. Less agreement was observed with the three tests in determining the infecting strain with canine sera, whereas in the bovine sera, the results clearly pointed to *L. pomona* serotype.

TABLE III. CAPILLARY AGGLUTINATION REACTIONS ON SERA FROM APPARENTLY NORMAL ANIMALS

ANTIGEN	SERUM TITER	SOURCE OF SERUM					
		HUMAN		CANINE		BOVINE	
		NO.*	%	NO.	%	NO.	%
L. icterohemorrhagiae	Neg.†	98	98	26	87	68	94
	1-8	2		2		1	
	1-16	0	2	2	13	2	6
	1-32	0		0		1	
	Total	100	100	30	100	72	100
L. canicola	Neg.	100	100	26	87		
	1-8	0		1			
	1-16	0	0	1	13		
	1-32	0		2			
	Total	100	100	30	100		
L. pomona	Neg.	97	97	21	70	50	69
	1-8	2		6		7	
	1-16	1		0		5	
	1-32	0	3	1	30	8	31
	1-64	0		2		1	
	1-128	0		0		1	
	Total	100	100	30	100	72	100

*No. = Number of sera.
†Neg. = No agglutination in serum diluted 1 in 8.

The patterns obtained by the 3 tests with heterologous antigens were quite different and cannot be explained simply by the assumption that they represented simple cross reactions. With serum 699, for example, the capillary agglutination and microscopic agglutination tests gave negative results with *L. icterohemorrhagiae* and *L. canicola* antigens, suggesting that both the agglutination tests were more highly serotype specific than the complement fixation test which gave positive reactions with both of the heterologous antigens. With serum 3,884, the complement fixation and the microscopic agglutination tests appeared serotype specific, whereas the capillary agglutination test revealed a higher titer with *L. pomona* than with *L. icterohemorrhagiae*, which was the presumptive infecting serotype.

The data indicate that the Leptospira antigens are exceedingly complex and suggest that the different tests may be measuring different types of antibody response.

TABLE IV. TITERS OBTAINED BY COMPLEMENT FIXATION, CAPILLARY AGGLUTINATION, AND MICROSCOPIC AGGLUTINATION WITH 3 SEROTYPES ON SERA FROM CASES OF LEPTOSPIROSIS

	SEROTYPE	L. ICTEROHEMORRHAGIAE			L. CANICOLA			L. POMONA		
	TECHNIQUE	COMP. FIX.	CAP. AGGL.	MICRO. AGGL.	COMP. FIX.	CAP. AGGL.	MICRO. AGGL.	COMP. FIX.	CAP. AGGL.	MICRO. AGGL.
Source of serum	Human 3,717	1-128	1-1,024	1-4,096	Neg.*	Neg.	Neg.	Neg.	1-1,024	Neg.
	Human 699	1-256	Neg.	Neg.	1-512	Neg.	Neg.	1-2,048	1-8,192	1-4,096
	Human 1,448	1-2,048	1-1,024	1-4,096	1-2,048	1-64	1-4,096	1-2,048	1-512	Neg.
	Human 1,315	1-128	Neg.	Neg.	1-64	Neg.	Neg.	1-256	1-128	1-512
	Human 3,884	1-512	1-2,048	1-8,192	Neg.	Neg.	Neg.	Neg.	1-8,192	Neg.
	Bovine 346	1-16	1-32	Neg.	1-64	1-16	1-64	1-2,048	1-1,024	1-2,048
	Bovine 347	1-32	1-32	1-64	1-128	Neg.	1-128	1-8,192	1-1,024	1-4,096
	Bovine 933	Neg.	1-32	Neg.	1-16	1-16	Neg.	1-32	1-256	1-256
	Bovine 929	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	1-128	1-256	1-256
	Canine 3,285	1-64	1-128	1-1,024	1-32	1-256	1-64	1-32	1-1,024	1-256
	Canine 3,649	1-256	1-512	1-1,024	1-128	Neg.	1-16	1-128	1-128	
	Canine 3,046	Neg.	1-16	1-64	1-128	1-512	1-512	Neg.	1-16	Neg.
	Canine 1,020	1-64		1-32	1-512		1-512	Neg.		Neg.
	Canine 670	1-1,024		1-4,096	1-8,192		1-1,024	1-512		1-1,024

*Neg. = No reaction in serum diluted 1 in 8.

The microscopic agglutination tests were performed independently by Mrs. Mildred Galton in a separate study. We are indebted to Mrs. Galton for permission to include her data in Tables IV and V.

TABLE V. TITERS OBTAINED BY COMPLEMENT FIXATION AND MICROSCOPIC AGGLUTINATION* TESTS ON SERIAL SAMPLES OF 5 PATIENTS WITH LEPTOSPIROSIS

SEROTYPE		L. ICTEROHEMORRHAGIAE		L. CANICOLA		L. POMONA	
TECHNIQUE		COMP. FIX.	MICRO. AGGL.	COMP. FIX.	MICRO. AGGL.	COMP. FIX.	MICRO. AGGL.
Patient "Pon"	9-28	1-512		1-1,024		1-4,096	
	10-16	1-256	Neg.†	1-512	Neg.	1-2,048	1-4,096
	11-18	1-256		Neg.		1-512	
	1-9	1-128	Neg.	1-64	Neg.	1-256	1-512
Patient "Sch"	9-17	1-512		Neg.		Neg.	
	9-27	1-16,384	1-4,096	Neg.	1-256	Neg.	1-128
	11-22	1-512		Neg.		1-16	
	1-10	1-64	1-1,024	1-8	Neg.	1-16	Neg.
Patient "H"	8-11	1-128	1-4,096	Neg.	Neg.	Neg.	Neg.
	8-18	1-2,048	1-8,192	Neg.	Neg.	Neg.	Neg.
	8-21	1-1,024	1-8,192	Neg.	Neg.	Neg.	Neg.
	8-24	1-32	1-8,192	Neg.	Neg.	Neg.	Neg.
Patient "S"	8-8	1-64	Neg.	Neg.	1-256	1-128	1-4,096
	9-5	1-8	Neg.	Neg.	1-1,024	1-8	1-8,192
Patient "McB"	11-2	1-2,048		1-512		1-1,024	
	11-22	1-64		1-8		1-32	
	12-1	1-32		Neg.		1-16	
	12-9	1-64		Neg.		1-64	
	2-1	1-64	1-512	1-64	Neg.	1-64	Neg.

*See footnote to Table IV.

†Neg. = No reaction in serum diluted 1 in 8.

Titers Obtained by the Complement Fixation and Microscopic Agglutination Tests on Sera Drawn at Intervals During the Course of Leptospirosis.—The data, presented in Table V, show the same phenomena in regard to cross reactions as the data given in the previous table. With Patient "Pon," the microscopic agglutination test gave reactions only with the *L. pomona* antigen, whereas the complement fixation test was positive with all 3 antigens. Both sera from Patient "S" reacted in both tests with *L. pomona* antigens, but a

cross reaction with *L. icterohemorrhagiae* antigen was noted only in the complement fixation test and a cross reaction with *L. canicola* only in the microscopic agglutination test. Four sera from Patient "H" showed high titers in both the complement fixation and microscopic agglutination tests with *L. icterohemorrhagiae*, and no cross reactions occurred with the two other antigens in either test.

TABLE VI. STABILITY OF L. ICTEROHEMORRHAGIAE DURING STORAGE

	TIME AFTER PREPARATION			
	1 DAY	1 MONTH	4 MONTHS	8 MONTHS
Treatment with 10% formaldehyde				
C. F. test performed with 0.01% $MgCl_2$ in barbital buffer	8/64*	16/64	16/64	16/64
C. F. test performed with 0.015% $MgCl_2$ in barbital buffer	4/64	8/64	16/64	16/64
Not treated				
C. F. test performed with 0.01% $MgCl_2$ in barbital buffer	32/64	64/64	128/16	
C. F. test performed with 0.015% $MgCl_2$ in barbital buffer	16/64	32/32	128/16	

*The numerator indicates the dilution of antigen used for testing (twice the dilution showing anticomplementary activity). The denominator gives the antibody titer obtained with a hyperimmune goat serum as tested with the antigen dilution indication in the numerator.

Stability of the Complement Fixation Antigen.—One of the difficulties inherent in the microscopic agglutination test is the tendency of formalinized antigens to become autoagglutinable on standing for various periods of time. Stoenner[3] reported that capillary agglutinating antigen was quite stable. Our experience with similar antigens made in this laboratory, particularly those prepared from other serotypes, has indicated that often there is some difficulty in determining titer, particularly in the higher serum dilutions; also, after storage, variations in sensitivity have been observed.

The stability of *L. icterohemorrhagiae* complement-fixing antigen was determined, at various intervals after preparation and during storage in the ice box, by measuring its anticomplementary activity and its combining power using hyperimmune serum. The effects of different concentrations of magnesium chloride in the buffer were determined, as well as the effects of preliminary treatment with formalin. The data, presented in Table VI, show that the antigens treated with formalin did not become increasingly anticomplementary over an eight-month storage period, and the results of complement fixation tests with hyperimmune serum showed that the combining power of the antigen had not changed over the prolonged storage period. The anticomplementary activities appeared to be less pronounced when the magnesium chloride content of the buffer was increased from 0.01 to 0.015 per cent. On the other hand, the nonformalinized antigens developed anticomplementary properties, making further dilution of the antigen necessary for the test procedure with a resulting drop in titer as measured by the hyperimmune serum.

DISCUSSION

The development of serologic methods as diagnostic tools in any infectious disease is dependent upon the accumulation of information concerning the nature and types of antibody response. In leptospirosis the picture is complicated further by the fact that the infecting organisms are rarely isolated and the serotypes causing the infections often are unknown. It has been observed that agglutinins to a heterologous serotype may appear in the early stages of infection, whereas high titers of antibodies to the organisms actually causing the infection appear later.[5-7]

The agglutination and agglutination-lysis tests are satisfactory in the hands of trained people but require microscopic darkfield examination and are tedious to perform if large numbers of sera are to be examined, especially if each serum is to be tested against a battery of antigens composed of a number of different serotypes. The sonic antigen, developed by the Army Medical Service Graduate School, represents a considerable improvement in serologic diagnosis because of the fact that a battery of only three antigens (*L. icterohemorrhagiae*, *L. hyos*, and *L. grippotyphosa*) is necessary for the detection of antibodies in the patient caused by any one of the eight serotypes known to cause infections in the United States.[8]

The data given in Table I of this paper indicate that the more easily prepared particulate antigens are more sensitive than the sonic antigens. The antigenic coverage of such antigens is quite widespread, as indicated by the previous work reported from this laboratory.[1] It is interesting to note that every serum specimen from a human case of leptospirosis fixed complement with the particulate *L. icterohemorrhagiae* antigen (Tables I, IV, V), every bovine serum from a case of leptospirosis reacted with the particulate *L. pomona* antigen (Table I, IV), and every canine serum reacted with the *L. canicola* antigen (Table I, IV).

Serologic data on an outbreak of *L. canicola* infection in Columbus, Georgia,[9] revealed that the complement fixation test tended to become positive early in the infection and then to become negative, whereas rises in agglutinin titers were observed later in the disease. However, the complement fixation tests on sera from the Columbus outbreak were performed with the sonic antigen, which has been shown to be less sensitive than the particulate antigen (Table I).

The present data are not sufficient to accept or reject the hypothesis that the complement fixation and the agglutination tests are estimates of similar antibody responses. Regardless of whether or not the complement fixation procedure is measuring a different antibody than that which yields a positive agglutination or agglutination-lysis reaction, it is suggested that the complement fixation test is a more satisfactory procedure for processing large numbers of sera submitted for the serologic diagnosis of leptospirosis. Although the data are insufficient to determine the sensitivity of the test in early cases

or in sera giving low titers with other tests, the absence of false positive reactions in normal humans and patients with other infections is a distinct advantage.

The particulate antigens are relatively easy to obtain in large quantity, their antigenic coverages are broad, they are relatively stable, and the end point, based on observation of extent of hemolysis, is not questionable as may be the case at times with agglutination reactions, especially when the antigens show some degree of autoagglutination.

Serologic studies, employing all three types of tests, on proved cases of leptospirosis in which the serotype causing the infection can be determined, are necessary to interpret the discrepancies now observed between the results obtained in the various types of test.

CONCLUSIONS

Further studies of complement fixation reactions with three Leptospira serotypes on sera from humans, cows, and dogs with leptospirosis are reported.

Normal humans and a limited number of patients with infections other than leptospirosis, including homologous serum jaundice and infectious hepatitis, gave negative reactions with the three antigens.

Comparative studies with data obtained by the capillary agglutination and microscopic agglutination techniques are presented.

It is suggested that the complement fixation test is the most practical test for use by reference diagnostic laboratories confronted with the problem of processing large numbers of sera submitted by physicians for serologic studies in leptospirosis.

REFERENCES

1. Schubert, J. H., Carrington, Lila, Conner, Evelyn, and Holdeman, Lillian: Whole Leptospira Suspensions as Antigen in the Complement Fixation Test for Leptospirosis, Am. J. Hyg. **63**: 1956.
2. Schubert, J. H.: A Simplified Procedure for the Preparation of Schüffner's Leptospira Medium, Pub. Health Lab. **13**: 67-70, 1955.
3. Stoenner, H. G.: A Capillary Tube Test for Leptospirosis, Am. J. Hyg. **57**: 316-327, 1953.
4. Kolmer, J. A., Spaulding, E. H., and Robinson, H. W.: Approved Laboratory Technique. New York, 1951, Appleton-Century-Crofts, Inc., p. 824 Fifth Edition.
5. Ward, Martha K.: Some Problems in the Laboratory Diagnosis and Control of Leptospirosis, Vet. Med. **49**: 147-152, 1954.
6. Gispen, R., and Schüffner, W.: Die Spaltung der klassichen *Leptospira icterohemorrhagiae s. icterogenes* in zwei biotypen. Z. Bakt., I. orig. **144**: 427, 1939.
7. O'Connell, P. T., and Broom, J. C.: Weil's Disease, a Cautionary Tale, Lancet **1**: 1088, 1952.
8. Gochenour, W. S., and Yager, R. H.: Classification of North American Leptospiras, Proc. Am. Vet. Med. Assoc. p. 178, 1952.
9. Ward, M. K., McDaniel, M. B., Tatum, H. W., Starr, L. E., and Williams, H. R.: An Epidemic of Canicola Fever in Man With the Demonstration of *Leptospira Canicola* Infection in Dogs, Swine, and Cattle. II. Laboratory Studies. (To be published).

Errata

In the article entitled "The Effects of Isotonic and Hypertonic Salt Solutions on the Renal Excretion of Sodium," by SOLOMON PAPPER, M.D., LAWRENCE SAXON, M.D., JACK D.

ROSENBAUM, M.D., and HENRY W. COHEN, M.D., which was published in the May issue of the JOURNAL, page 776, in the paragraph under "Methods," the abbreviation for microequivalent should be changed to milliequivalent as follows: line 3, "140 meq. of sodium"; line 6, "338 meq. of sodium"; line 8, "154 meq. per liter"; line 9, "850 meq. per liter"; line 11, "25 meq. per liter."

In the article entitled "Antibacterial Activity of Streptonivicin and Cathomycin, Two New Antibiotics" by WILFRED F. JONES, JR., M.D., ROGER L. NICHOLS, M.D., and MAXWELL FINLAND, M.D., which appeared in the May issue of the JOURNAL, pages 783-792, the generic name of the antibiotic in the Addendum on page 792, next to last line, should read "novobiocin."